Study of the transferability of microsatellite markers derived from bread wheat (T. aestivum) or rice (O. sativa) ESTs (EST-SSRs) to their close and wild relative and evaluation of their potential for the organisation of genetic resources in the grass family

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UNIVERSITE BLAISE PASCAL
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UNIVERSITE D'AUVERGNE

Année 2006

ECOLE DOCTORALE
DES SCIENCS DE LA VIE & DE LA SANTE

Nº d'ordre 437

T H E S E

Présentée à l'Université Blaise Pascal
pour l'obtention du titre de

DOCTEUR D'UNIVERSITE

(Spécialité : Biologie Moléculaire Végétale)

Soutenance le 23 mai 2006

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Président :	P. NICOLAS
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U.M.R. INRA/UBP Amélioration et Santé des Plantes – INRA de Clermont-Ferrand

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Remerciements

Cette thèse a été réalisée avec l'aide d'une bourse de China Scholarship Council (CSC)

Je tiens tout d'abord à remercier Michel Beckert et Gilles Charmet, directeurs successifs de la station d'Amélioration des Plantes de l'INRA de Clermont-Ferrand, pour leur accueil au sein de leur établissement.

Mes remerciements sont adressés à Michel Bernard et Catherine Feuillet qui m'ont accueillie dans leur équipe et pour leur critique des publications.

Mes remerciements sont particulièrement adressés à Pierre Sourdille qui m'a encadrée au laboratoire et m'a aidé pour la rédaction de ma thèse et des publications avec beaucoup de patience et de façon très constructive. Nos échanges scientifiques sur les marqueurs microsatellites ont été passionnants et fructueux.

J'adresse également toute ma reconnaissance à Sylvie Bernard pour son aide pour la prononciation du français et à Philippe Leroy qui m'a initiée aux blast contre les séquences génomiques chez le riz et le blé. Merci encore à Catherine Ravel pour m'avoir aidée dans les analyses statistiques et la construction des arbres phylogénétiques et à François Balfourier pour ses conseils dans les analyses de diversité et pour la fourniture de toutes les espèces cultivées ou sauvages de graminées. Merci enfin à Christiane, Jérome et Pierre B pour leurs précieux conseils et à Gilles B et Nicolas pour m'avoir aidé à analyser les produits PCR sur Genescan et Genotyper

Une pensée toute particulière à tous ceux qui sont passés au labo BM et Génotypage, qui sont partis ou y sont encore : Bouzid, Delphine B, Cécile, Vincent, Nathalie N, Valérie, Fatou, Elisabeth, Delphine R, Etienne, Walid, Cyril, Caroline, Nathalie M, Séverine, Lydia, Pierre D, Jacqueline et Stéphane.

Merci à Pascal pour avoir arrangé beaucoup de choses et à Jeff pour le coup de main avec le PC et les problèmes d'imprimante.

Merci à ma famille et surtout à ma mère et mon père pour m'avoir encouragée et soutenue.

Enfin, merci à mon mari et mon fils pour leur soutien, et surtout à mon fils pour m'avoir accompagnée pendant tout ce temps en France.

Abbreviations

DNA：DeoxyriboNucleic Acid
AFLP: Amplified Fragment Length Polymorphism
CDS: Coding DNA Sequence
CTAB: Cetyl Trimethyl Amonium Bromide
CIMMYT : Centro International de Mejoranniento de Maiz Y Trigo (Mexique)
BLAST: Basic Local Alignment Search Tool
EDTA: Ethylene Dinitrilo Tetraacetic Acid
EST: Expressed Sequence Tag
EST-SSR: Simple Sequence Repeats derived from Expressed Sequence Tag
FAO: Food and Agriculture Organization
H_2O_{UP}: ultra pure water
ISSR: Inter Simple Sequence Repeats
ITMI: International Triticeae Mapping Initiative
kb: kilobase
ORF: Open Reading Frame
MAS: Marker Assisted Selection
NIRS: Near Infrared Spectroscope
pb: pair of bases
PCR: Polymerase Chain Reaction
PIC: Polymorphism Information Content
QTL: Quantitative Trait Locus
RAPD: Random Amplified Polymorphic DNA
RFLP: Restriction Fragment Length Polymorphism
SCAR: Sequence-Characterized Amplified Region
SDS: Sodium Dodecyl Sulfate
SNP: Single Nucleotide Polymorphism
SSLP: Simple Sequence Length Polymorphism
SSCP: Simple Sequence Conformation Polymorphism
SSR: Simple Sequence Repeat
STS: Sequence Tagged Site
TBE: Tris Borate EDTA
TE: Tris EDTA
TEMED: Tetra Methyl Ethylene Diamine
TIGR: The Institute for Genomic Research
5’-UTR: 5’- UnTranslated Regions
3’-UTR: 3'- UnTtranslated regions

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INTRODUCTION

Introduction

Since humans have domesticated and cultivated the plant species that were necessary for their own or cattle feedings or for their material needs, they have been confronted to numerous plant development harmful events. Those events are abiotic stresses such as drought or soil salinity as well as biotic stresses like devastating insects or diseases. In order to face these constraints, several cultural practices aiming at correcting the environment such as irrigation, fertilizer supplies, or phytosanitary treatments were developed. At the same time, knowledge in plant genetics and especially cereals that have dramatically increased since the beginning of the 20th century, have been extensively exploited in order to genetically improve cultivated crops (Green Revolution during the sixties). Especially, criteria that are the most largely taken into account in genetic breeding are yield, environment adaptation (earliness, drought resistance…) and disease resistances.

Until now, the cereals world production has covered the human needs, even outpacing population growth leading to surpluses in the past twenty years. However, the demographic increase that is estimated for the next 50 years will induce a simultaneous increase in the world cereal demand for feeding. If cultivating more and more lands has been the traditional answer to address the growing needs of the population, this will no longer be the case because of the decrease of agricultural land areas, especially in the developing countries, leading thus probably to stock depletions.

In addition, agriculture has also now to face ecological constraints. Intensive agriculture has induced huge modifications of the environment (deforestation, suppression of the hedges and ditches…) leading in soil degradation and in flooding. Also, intensive use of fertilizers, herbicides, insecticides and fungal treatments has conducted simultaneously to deplete the animal as well as floral genetic variability, to pollute phreatic water and to contribute to the eutrophy of lakes and rivers.

Thus, improvement of cereal yield in the context of sustainable agriculture has to be reached in the next decades to meet human needs by 2050. Significant advances in the understanding of the plant biology as well as in the management and exploitation of genetic resources must be achieved to face this challenge. Concerning this latter point, genetic variability in cereals and especially in wheat is extremely extended but remains largely underexploited. This is due: (1) to the poor knowledge of the capacities of these

resources in terms of biotic and abiotic resistances as well as their potential for bread making quality or yield performances; (2) to the fact that they are bearing many traits of low agronomical interest such as plant height, lodging susceptibility, free threshing, hulled kernels… Because of this, low attentions have been paid on the study of wheat related species especially in genomic areas while genomics has exploded in wheat in the last five years leading to better marker-assisted selection and to the positional cloning of a number of genes of agronomic interest.

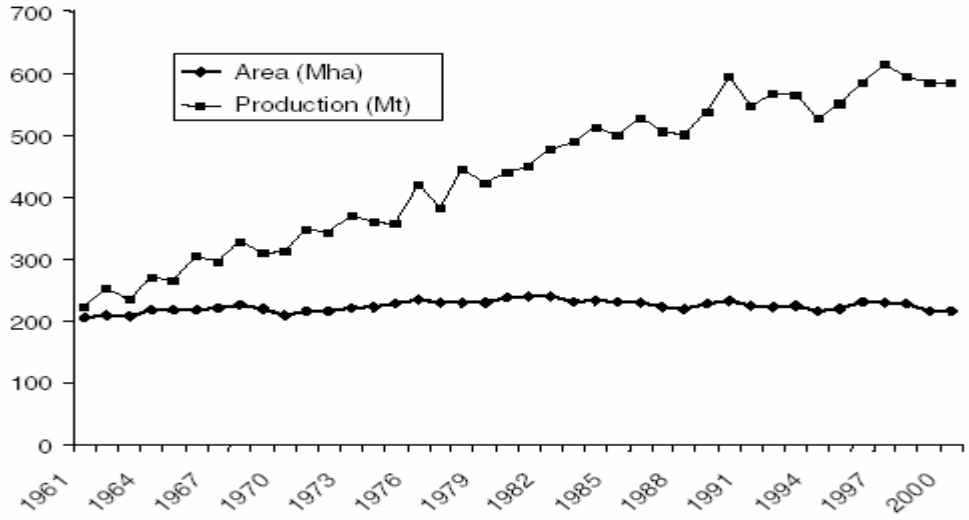
The first aim of the thesis was to develop efficient genomic tools to study a wide range of wheat related species. Especially, we decided to focus our efforts in the development of molecular markers that can be used on wheat as well as on a large range of wild and cultivated wheat-related species that can be of interest for wheat genetic improvement. These markers would further be used to follow introgressions of genes of agronomical interest issued from these species, and to reduce the linkage drag of unfavourable alleles that they carry. The second aim was to use these markers to study the genetic variability existing within these species in comparison to wheat, in order to see whether they can be used as source of new alleles. We also assessed the relationships between and within these species. All this work will support the research towards the improvement of wheat and a better understanding of the organisation, function and evolution of the wheat genome.

BIBLIOGRAPHY

Tab 1-1. World wheat production (million tonnes)

	2004 estimate	2005 forecast	2005 over 2004 (%)
Asia	**254.1**	**264.0**	**3.9**
Far East	185.9	192.7	3.7
Near East in Asia	45.1	46.4	2.9
CIS in Asia	22.1	23.8	7.7
Africa	**23.1**	**21.5**	**-6.9**
North Africa	17.5	16.7	-4.6
Eastern Africa	3.6	2.4	-33.3
Southern Africa	1.9	2.3	21.1
Central America & Caribbean	**2.4**	**3.0**	**25.0**
South America	**25.3**	**21.6**	**-14.6**
North America	**84.6**	**79.4**	**-6.1**
Europe	**216.7**	**205.2**	**-5.3**
EU 25	135.1	126.0	-6.7
CIS in Europe	64.7	63.0	-2.6
Oceania	**20.7**	**20.3**	**-1.9**
World	**626.8**	**614.9**	**-1.9**
Developing countries	280.2	283.2	1.1
Developed countries	346.6	331.8	-4.3

Fig1-1. Wheat production worldwide compared with the area sown to wheat from 1961 to 2000.



Chapter I: Bibliography

1. Hexaploid wheat

Small grain cereals offer the decisive advantage of constituting supplies that can be stored as grains, which have a high nutritional value due to their starch as well as protein contents. In addition, they can be easily transformed by cooking under numerous forms. First archeological evidences of cereal harvests date about 10,000 years ago and were found in the Middle-East, in the "Fertile Crescent" (Feldman, 1976) where einkorn (*T. boeoticum*) and wild durum species (*T. dicoccoides*) were subjected to gathering. Among the cereals, bread wheat (*Triticum aestivum* L.) occupies arguably the most important place. It appeared in the same region about 8,000 years ago and was one of the first domesticated crops. Then it was dispersed all over the world from Greece and Europe and has served as staple food for the major civilizations in Europe, West Asia and North Africa. At the present time, wheat remains the most important food source for humans and one of the most important merchandise for economical exchanges.

1.1 Economical importance of wheat

Wheat is grown as a commercial crop in 120 countries on an area of 217 million hectares in 2004 (FAO source) which represents about one third of the total cereal-cultivated areas. Wheat is also the most productive among the cereals and the combined harvests in 2004 were 627 million metric tonnes. The major producers in 2004 were China, European Union (25 countries), India, U.S.A., Russian Federation, Canada and Australia. The world wheat production in 2005 is expected to be 612 million tonnes, 15 million tonnes lower than the record in 2004 but still well above than the average of the past five years (Tab. 1-1) (FAO; http://www.fao.org/).

Improvement of cultural practices and genetic breeding since the beginning of the 20th century largely contributed to increase the world yields from 0.9 t/ha in 1900 to 2.9 t/ha in 2004. For example, since the early 1960s there has been little increase in the area sown with wheat, but over the same period, yields have increased almost 3-fold (Fig. 1-1). However, yields remain very different among the countries and if they can reach a mean of 7.8 t/ha in the Netherlands, they are only of 0.34 t/ha in Somalia.

Wheat also takes the first place in the international agricultural trade of cereal productions.

Fig 1-2. Wheat import by region

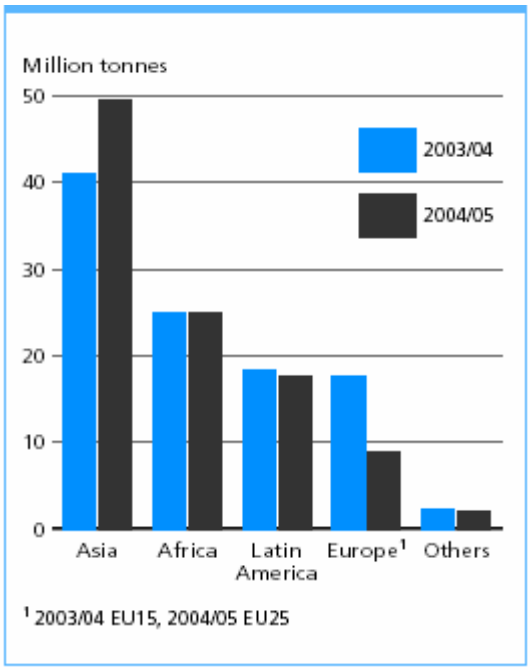


Fig 1-3. Wheat export price (US No.2 Hard Winter, Gulf)

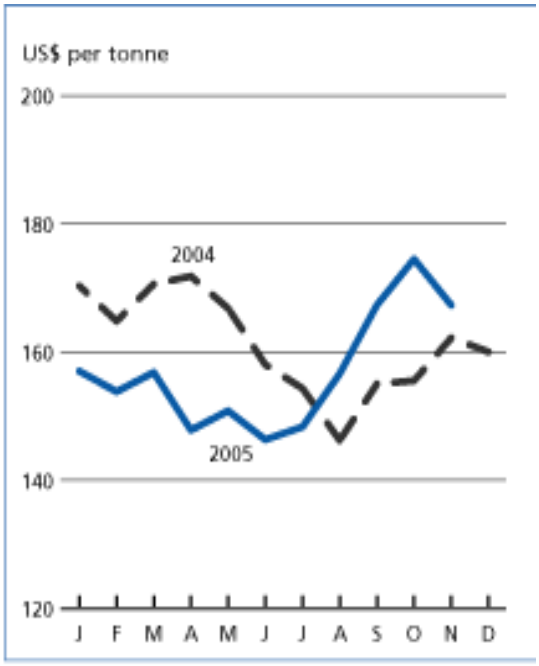
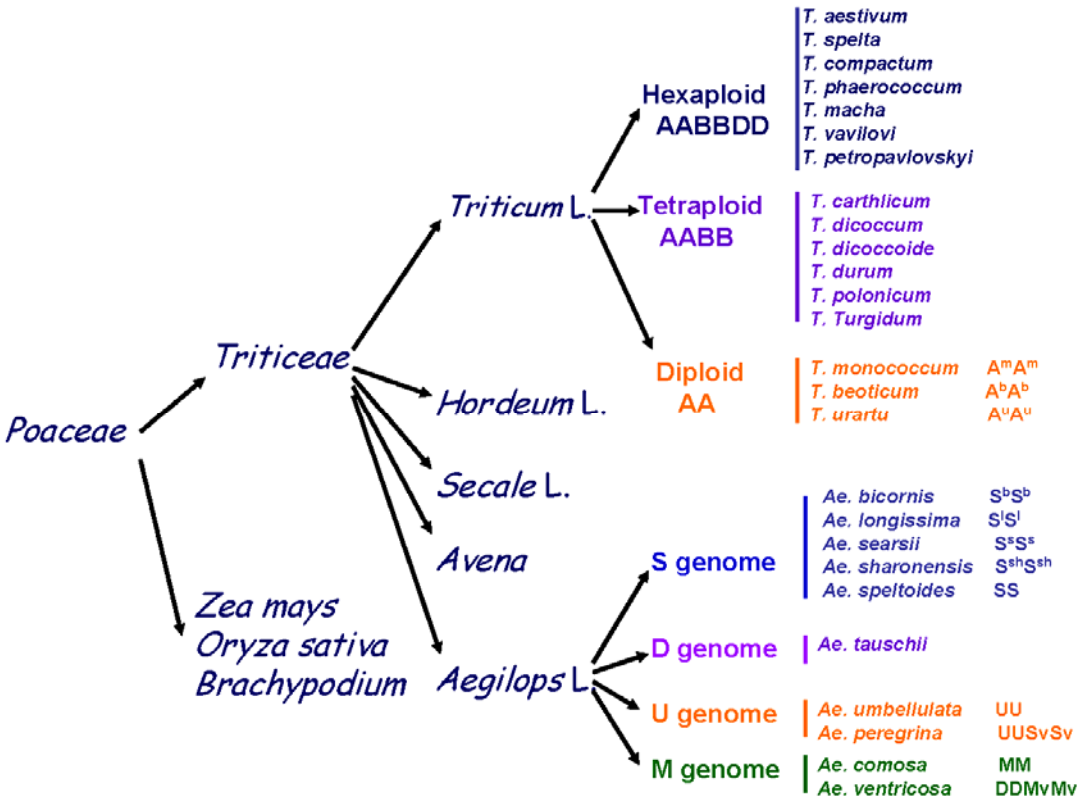


Fig 1-4: Close and wild related species of *T. aestivum*



World trade in wheat in the current 2004/2005 marketing year is now forecast to reach 103 million tonnes (FAO; http://www.fao.org/). Fig. 1-2 and 1-3 indicate the situation of wheat import of each region in the whole world and the price of wheat export in 2004 and 2005, respectively.

Wheat is primarily grown for its grain which is mainly used in bakery but it has other uses in the livestock feed and industry. Bread wheat can be roughly classified into several types, based on the growth habit (spring *vs* winter) and the hardness of the kernel endosperms (hard vs soft). The hard types of bread wheat often show high protein contents, especially gliadins and glutenins. They are mainly utilized for the production of a large variety of leavened and flat breads. The high level of these protein fractions in the flour imparts elasticity to bread dough and allow it to expand during leavening and baking. On the contrary, soft wheats are generally low in protein content, and are thus mostly milled into flour for use in a wide variety of bakery products such as cakes, pastries, and unleavened breads.

1.2 Classification of the hexaploid wheat

Botanically, wheat (*Triticum aestivum* L. em Thell) belongs to the sub-family of the *Pooideae* within the grass family (*Poaceae*) which also includes the sub-families of the *Bambusoideae* that comprises rice (*Oryza sativa*) and of the *Panicoideae* that comprises maize (*Zea mays*), sorghum (*Sorghum bicolor*) and sugar cane (*Saccharum officinarum*). Within the *Pooideae* sub-family, wheat belongs to the *Triticodeae* super-tribe and to the *Triticeae* tribe. This latter tribe includes three important sub-tribes: the *Triticineae* with the *Triticum* and *Aegilops* species, the *Secalineae* with rye (*Secale cereale*) and the *Hordineae* with barley (*Hordeum vulgare*). Over two dozen individual species have been characterized as members of the genus *Triticum* among which only *T. monococcum* L., *T. turgidum* L., *T. aestivum* L. are widely cultivated and only the two latter are common. Relationships between *T. aestivum* and its close and wild relatives are listed in Fig. 1-4.

It was early demonstrated that wheats formed polyploid series (Sakamura, 1918). The diploid species contain eight distinct genomes that were given a letter as names often followed by an additional letter representing the sub-species: A (*T. monococcum ssp. boeoticum*, A^b; *ssp. monococcum*, A^m; *ssp. urartu*, A^u), C, D, M, N, S (*Sitopsis* section: *Ae. speltoides*, S^s; *Ae. bicornis*, S^b; *Ae. longissima*, S^l; *Ae. sharonensis* S^{sh}), T and U. Two of the genomes found in polyploid wheats were given new names, B and G, because their

Fig. 1-5: Phylogenetic relationship of *T. aestivum*

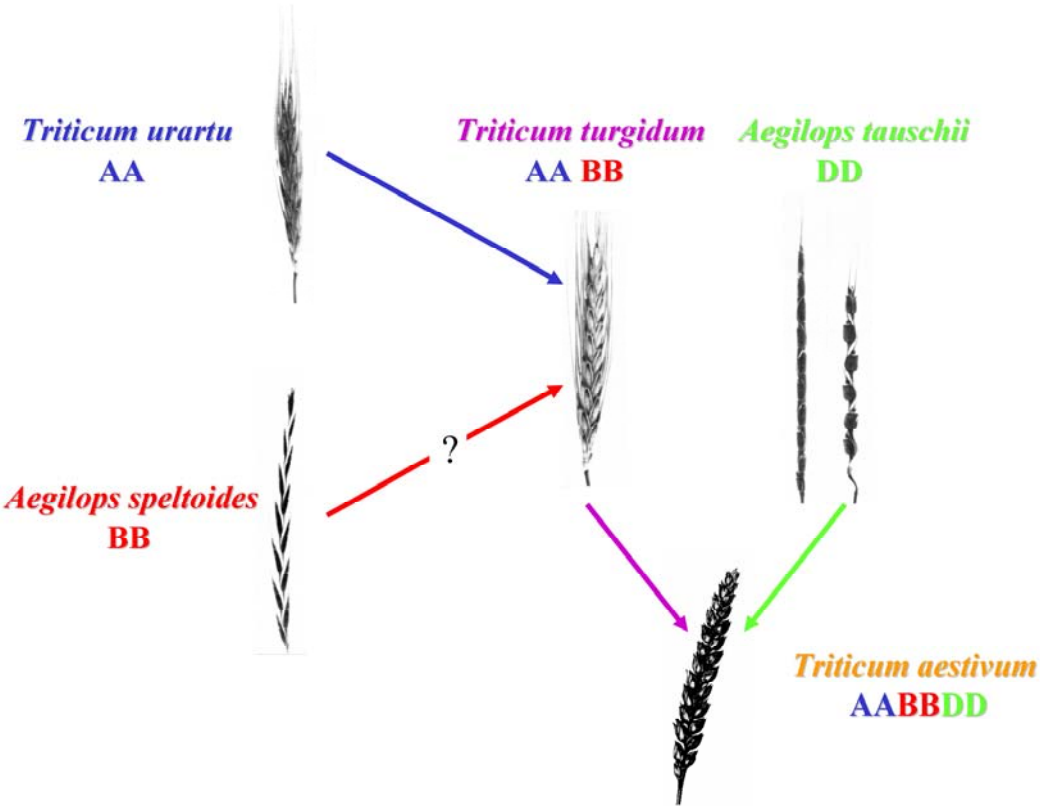
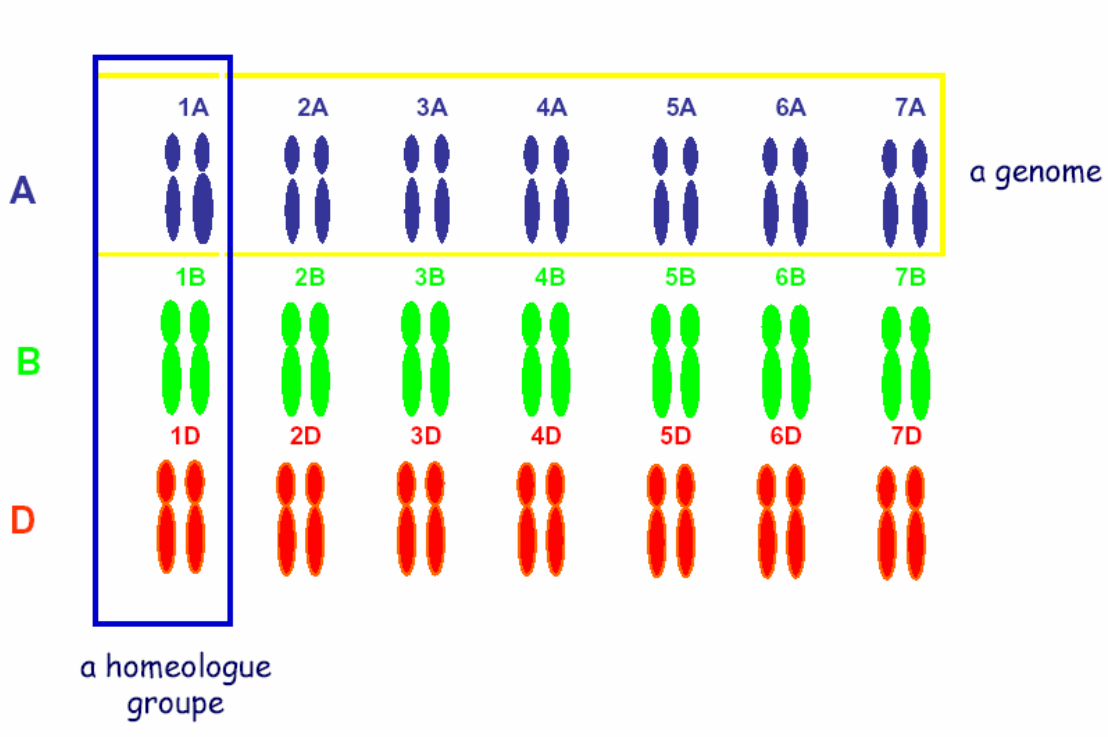


Fig 1-6: Chromosome structure of *T. aestivum*



diploid progenitors were not known. Some of the diploid species contributed to the genomes of polyploid species. The *Triticum* and *Aegilops* species gather diploid as well as polyploid species among which hexaploid bread wheat (*Triticum aestivum*) occupies the most important place of the *Triticeae* tribe.

1.3 Origins of bread wheat

Hexaploid bread wheat (*Triticum aestivum* L.em.Thell. 2n = 6*x* = 42) is an allopolyploid species which arose under cultivation 8,000 years ago from hybridization, followed by spontaneous chromosome doubling, between *T. turgidum* ssp. *dicoccum* and the diploid goatgrass *Aegilops tauschii* ssp. *strangulate* (2n= 6*x* =14, DD-genome; McFadden et al. 1946; Jaaska 1980). Early cytogenetic studies suggested that the A genomes of the polyploids were contributed by *T. monococcum* (Sax 1922; Lilienfeld and Kihara 1934) but more recent studies evidenced that *T. urartu* was the real donor of the A genome (Natarajan et al. 1974; Huang et al. 2002). The origins of the B genome remain uncertain and controversial (Kerby and Kuspira, 1987). At least six different diploid *Aegilops* species from the *Sitopsis* section were proposed as possible source of the B genome. Other studies suggest a possible polyphyletic origin of the B genome. Two different allotetraploids with a common A genome (AAXX and AAYY) could have hybridized and within their descent, the X and Y genomes could have rearranged and gave rise to the actual B genome. However, *Ae. speltoides* is at the present time the most likely living relative of an extinct or yet to be discovered B genome donor species (Fig. 1-5, Sarkar and Stebbins 1956; Riley et al. 1958; Rees and Walters 1965; Natarajan and Sharma 1974; Chen et al. 1975; Jaaska 1980; Hassan and Gustafson 1996, Maestra and Naranjo 1998).

1.4 Genetic specificity of bread wheat

The wheat genome is hexaploid and is made by the juxtaposition of three simple genomes named A, B and D also called homoeologues. DNA is organized into 21 pairs of chromosomes (Fig. 1-6), seven pairs belonging to each of the A, B and D genomes (Sears 1954; Okamoto 1962). Within each diploid genome, the chromosomes are designated from 1 to 7. The size of each diploid genome varies between 4,500 and 6,000 mega bases (Mb, Bennet and Leitch, 1995) depending on the species. The bread wheat genome size was thus estimated to be 16,974 Mb (1C, Bennet and Smith, 1991) which represents 4, 40 and 130 times more than the human, rice and *Arabidopsis thaliana* genomes respectively. Wheat

genome is very complex and consists of unique or low-copy sequences surrounded by regions of highly repetitive DNA which represents about 70-80% of the genome (Flavell et al., 1977; Vedel and Delseny, 1987; Wicker et al., 2003). Although early studies indicated gene clustering in gene-rich islands located in distal parts of the chromosomes (telomeres), it is now believed that the islands are dispersed throughout the whole length of the chromosomes (Akhunov et al., 2003). Repetitive DNA mainly consists of transposable elements (e.g. *Fatima*, *Caspar* and *Angela*, Sabot et al., 2005) but Simple Sequence Repeats (SSRs) located in non-coding as well as in coding sequences are also frequent.

Fig 1-7: Examples of different types of SSRs

a. Perfect repeats without interruptions

GAGAGAGAGAGAGAGAGAGAGAGAGAGAGA

b. Imperfect repeats with one or more interruptions

GAGAGAGAGAGAGAG**C**GAGAGAGAGAGAGAGA

c. Compound repeats with adjacent tandem simple repeats of a different repeat:

Perfect compound repeats: GAGAGAGAGAGAGAGAGTGTGTGTGGTGTGTGTG

Imperfect compound repeats: GAGAGAGAGAGAGAG**GG**GTGTGTGGTGTGTGT

2 Simple Sequence Repeats (SSRs)

2.1 Definition and classification

Microsatellites, also referred to as Simple Sequence Repeats (SSRs), Variable Number of Tandem Repeats (VNTRs) or Variable Simple Sequence Motif (VSSM), were first described in eukaryotes in the early eighties (Hamada et al. 1982). They are defined as stretches of DNA, which consist of only one or a few (maximum of six) tandemly, repeated nucleotides, such as poly (A/T) or poly (GT/CA) of less than 100 bp in length with a minimal length of 12 bp (Tautz and Rentz, 1984; Tautz 1993). These types of simple sequence were shown to be repetitive and interspersed in many eukaryotic genomes (Tautz et al. 1986). Several other types were found by sequencing eukaryotic DNA. They were reported in the genome of diverse species (Hamada et al. 1982; Tautz and Rentz 1984; Greaves and Patient 1985; Dover and Tautz 1986) and have been implicated in a range of functions including gene regulation (Wang et al. 1979; Weintraub and Groudine 1976; Hentschel 1982; Shen et al. 1981), signals for gene conversion and recombination (Shen et al. 1981; Goodman 1996; Jeffreys et al. 1985), and the replication of telomeres (Blackburn and Szostak, 1984).

SSRs are divided into different categories according to their composition (Fig 1-7; Weber 1990): perfect, imperfect or compound repeats. They are also classified into two classes based on their origins. Some are developed from enriched genomic DNA libraries (Edwards et al. 1996; Ostrander et al. 1992) and are thus named genomic SSRs (g-SSRs). Most of them have neither genic function nor close linkage to coding regions (Metzgar et al. 2000), and their developing process is very tedious and expensive. The second class of SSRs is derived from EST sequences originating from the expressed regions of the genome and is named EST-SSRs. In general, EST-SSR markers produce high quality patterns, but give a lower level of polymorphism compared to that from genomic SSRs (Holton et al. 2000; Thiel et al. 2003). An important feature of the EST-SSR markers is that they can be rapidly developed from the EST databases at low cost, and due to their existence in expressed regions, this will increase the efficiency of selecting genes of interest through marker assisted selection (MAS).

2.2 Advantages of SSRs compared to other markers

The numerous advantages of the microsatellites have been well-documented (Morgante

and Olivieri 1993; Rafalski and Tingey 1993; Powell et al. 1996). One of the main advantages of the microsatellites compared with other markers is their highly polymorphic rate due to the variability of the number of repeats at a given locus (Poulsen et al. 1993; Schmidt et al. 1993; Thomas and Scott 1993; Senior and Heun 1993; Becker and Heun 1995; Rongwen et al. 1995). However, they remain sufficiently stable to avoid somatic variations (Barret 1993). The polymorphism can be evaluated through the polymorphism information content value (PIC value), a criterion that was first introduced by Nei et al. (1973) in human genetics. These are also mainly co-dominant markers with a Mendelian inheritance, which can reveal homozygous as well as heterozygous lines and which can be used on a wide range of segregating population (Morgante and Olivieri 1993). In addition, they show a high reproducibility compared to other markers such as RAPDs. When polyploid plants like rapeseed and wheat are considered, microsatellites are also frequently specific of the genome and give a single signal compared to RFLPs (Poulsen et al. 1993; Röder et al. 1995). Finally, they can be easily automated and used on high throughput genotyping platforms (Nicot et al. 2004).

2.3 SSRs within genomes

2.3.1 Abundance in genomes

Abundance and polymorphism of the SSRs, especially of the dinucleotides (TG)n, (AC)n and (AT)n was demonstrated in human and other eukaryotes (Weber and May 1989; Tautz 1989; Litt and Luty 1989). In general, plants have a lower proportion of sequences that account for SSRs than do vertebrates but a higher proportion compared with invertebrates and fungi. The range for plants is between 0.85% (*Arabidopsis*) and 0.37% (maize) of the genome (Morgante et al. 2002), whereas estimates for the fish species *Tetraodon nigroviridis* and *Fugu rubipres* are respectively 3.21% (Crollius et al. 2000) and 2.12% (Elgar et al. 1999), 1.07% for human chromosome 22, 0.21% for *Caenorhabditis elegans* and 0.30% for *Saccharomyces cerevisiae* (Toth et al. 2000). Within higher plants, Morgante and Olivieri (1993) estimated that the frequency of the microsatellites was one every 50 kb which contrasts with what is observed in human, with an estimated average density of one SSR every 6 kb (Beckman and Weber. 1992). Only few SSRs were detected within the organellar genome (Wang et al. 1994). The different types of SSRs exist in different density throughout the genome with slight variations according to the species. In primates, mononucleotides (mainly, poly (A/T) tracts) are the most copious classes of SSRs (Toth et

al. 2000; Wren et al. 2000). Dinucleotide (AT)n motifs were found to be rare within animal genomes while (CA)n were the most common SSRs (Moore et al. 1991). Most of the SSRs (48-67%) found in plant species are dinucleotide repeats while mono- and tetranucleotide repeats are the least common (Wang et al. 1994; Schug et al. 1998). A mean of one SSR every 64.6 kb and 21.2 kb was detected for monocotyledons and dicotyledons species respectively. However, frequency can vary from one every 3 kb in barley (Becker and Heun 1995) to one every 80 kb in rice (Panaud et al. 1995). At the centimorgan level, the SSRs provide thus an important source of markers for genetic mapping in eukaryotes. The utilization of microsatellites as genetic markers for a global approach of the genetic mapping in eukaryotes was proposed (Beckman and Soller 1990) and they have been thus extensively used to elaborate genetic maps in human (Weissenbach et al. 1992), pig (Rohrer et al. 1994) and other mammals and plants.

2.3.2 Dispersion within genomes

Microsatellites were found to be widely and randomly distributed in coding as well as non coding regions of the genome. A high proportion of SSRs is associated to *Alu* and *SINE* elements in primates and human (Arcot et al. 1995; Nadir et al. 1996) and thus forms a rather large portion of non-coding DNA regions. It was also demonstrated that they are frequently associated with retro-transposons and other dispersed repetitive elements in barley (Ramsay et al. 1999) as well as in other plant species (Wang et al. 1994). Di- and tetranucleotide SSRs are more frequent in non coding regions compared with trinucleotides. Dinucleotides are about 20 times more frequent in random genomic clones of Norway spruce (*Picea abies*, Scotti et al. 2000) compared to expressed sequences while Morgante et al. (2002) reported that all SSR types except tri- and hexa-nucleotides are significantly more frequent in the non coding fraction of six plant species compare to 25,762 predicted protein-coding sequences. In some cases, it was suggested that they could be clustered in some region of the genome (Condit and Hubbel 1991; Panaud et al. 1995; Arens et al. 1995).

Other evidences show that dinucleotide motifs seem to be located closer to the coding regions, in the 5’ UTRs or in the introns while one third of the trinucleotide motifs are located in the coding fraction of the genes (Morgante and Olivieri 1993). Introns have a similar repeat-unit profile to genomic DNA with various biases depending on species and

Tab 1-2: Various types of microsatellites (from Jin et al. 1994)

Mononucleotide motif (2):

A	C

Dinucleotide motif (4):

AC	AG	AT	CG

Trinucleotide motif (10):

AAC	AAG	AAT	ACC	ACG	ACT	AGC	AGG
ATC	CCG						

Tetranucleotide motif (33):

AAAC	AAAG	AAAT	AACC	AACG	AACT	AAGC	AAGG
AAGT	AATC	AATG	AATT	ACAG	ACAT	ACCC	ACCG
ACCT	ACGC	ACGG	ACGT	ACTC	ACTG	AGAT	AGCC
AGCG	AGCT	AGGC	AGGG	ATCC	ATCG	ATGC	CCCG
CCGG							

Pentanucleotide motif (102):

AAAAC	AAAAG	AAAAT	AAACC	AAACG	AAACT	AAAGC	AAAGG
AAAGT	AAATC	AAATG	AAATT	AACAC	AACAG	AACAT	AACCC
AACCG	AACCT	AACGC	AACGG	AACGT	AACTC	AACTG	AACTT
AAGAC	AAGAG	AAGAT	AAGCC	AAGCG	AAGCT	AAGGC	AAGGG
AAGGT	AAGTC	AAGTG	AATAC	AATAG	AATAT	AATCC	AATCG
AATCT	AATGC	AATGG	AATGT	AATTC	ACACC	ACACG	ACACT
ACAGC	ACAGG	ACAGT	ACATC	ACATG	ACCAG	ACCAT	ACCCC
ACCCG	ACCCT	ACCGC	ACCGG	ACCGT	ACCTC	ACCTG	ACGAG
ACGAT	ACGCC	ACGCG	ACGCT	ACGGC	ACGGG	ACGTC	ACTAG
ACTAT	ACTCC	ACTCG	ACTCT	ACTGC	ACTGG	AGAGC	AGAGG
AGATC	AGATG	AGCAT	AGCCC	AGCCG	AGCCT	AGCGC	AGCGG
AGCTC	AGGAT	AGGCC	AGGCG	AGGGC	AGGGG	ATATC	ATCCC
ATCCG	ATCGC	ATGCC	CCCCG	CCCGG	CCGCG		

motifs (Toth et al. 2000). Despite the fact that numerous SSRs exist in the open reading frames (ORFs) of higher eukaryotes including *Drosophila*, *Caenorhabditis elegans*, mammals, humans, plants and yeast (Toth et al. 2000; Katti et al. 2001; Kantety et al. 2002; Morgante et al. 2002), their occurrence in coding regions seems to be limited by non-perturbation of ORFs (Metzgar et al. 2000). In human cDNA databases, more than 92% of the predicted SSR polymorphisms within coding sequences have repeat-unit sizes that are a multiple of three (Wren et al. 2000). Thus, in many species, exons contain rare dinucleotide and tetranucleotide SSRs, but have many more trinucleotide and hexanucleotide SSRs than other repeats. Trinucleotide repeats show approximately a two fold greater frequency in exonic regions than in intronic and intergenic regions in all human chromosomes except the Y chromosome (Subramanian et al. 2003).

Within transcribed regions, UTRs harbor more SSRs than the coding regions themselves (Wren et al. 2000; Morgante et al. 2002). In *Arabidopsis*, the 5'-UTRs, exhibit a strong bias toward AG/CT contrary to human where 3'-UTRs show a bias toward AC/GT. Also, the 5'-UTRs contained more trinucleotide repeats than the 3'-UTRs in humans (31.1% vs. 4.6%; Stallings 1994; Wren et al. 2000) as well as in barley (67% vs. 26%; Thiel et al. 2003).

2.3.3 Composition

Because of permutations that give complementary repeats, only 501 different types of SSR motifs can be encountered from the mono- to hexa-nucleotides (Tab 1-2 derived from Jin et al. 1994). For example, the (AAC)n motif includes AAC, ACA and CAA repeats for the forward sense and GGT, TGT and TTG repeats for the reverse sense. There are thus two mono-, four di-, 10 tri-, 31 tetra-, 98 penta- and 356 hexa-nucleotide motifs.

Contrary to plants, mononucleotides are the most frequent in primate genomes (Toth et al. 2000) and there is a prevalence of A/T repeats (11.8%) compared with G/C repeats (0.7%) in human coding regions (Olivero et al. 2003). However, the majority of SSRs (48 – 67%) found in many species are dinucleotides (Wang et al. 1994; Schug et al. 1998). Compared with animals, it was noticed that in higher plants, there is a lack of (CA)n repeats while (AT)n are the most frequent SSRs (Akkaya et al. 1992; Morgante and Olivieri 1993; Lagerkrantz et al. 1993; Bell and Ecker 1994). This pattern may be related to higher frequencies of certain amino acids in plants than in animal (Toth et al. 2000). The motif (GC)n was only slightly detected in plants (Becker and Heun 1995; Morgante and Olivieri

1993). On the contrary, the (GA)n repeats are more abundant than the (CA)n repeats which seems to be characteristic of the plant genomes (Condit and Hubbel 1991). Exons and ESTs show higher frequency for GA/CT repeat than for AT repeat in *Arabidopsis thaliana* and cereals (Morgante et al. 2002; Kantety et al. 2002). However, the proportion of the different types of microsatellites depends on the species. For example, in *Arabidopsis*, frequencies of the (CA)n and (GA)n repeats are lower compared to what is estimated in other plants (Bell and Ecker 1994). In rice, microsatellites (GA)n are the most frequent (Panaud et al. 1995). The proportion of dinucleotide repeats was greater among genomic DNA than among EST, and this proportion increased with longer SSRs (La Rota et al. 2004).

Concerning the trinucleotide motifs, microsatellites (TAT)n are the most frequently detected in animals as well as in plants (27.5%) followed by (TCT)n motif (25%; Morgante and Olivieri 1993). However, similarly to the dinucleotides, frequency varies among the species. In barley, the (AGC)n and (CCT)n motifs are the most frequent (Becker and Heun 1995) while in rice, it is the (TTG)n motif (Panaud et al. 1995). Trinucleotide repeats are significantly more abundant in EST sequences compared to genomic sequences. In the animals, (AGC)n repeat is the most common motif (40.9% - 60.9%) while in plants, monocotyledons are richer in GC-rich trinucleotide repeats compared to dicotyledons (Cordeiro et al. 2001; Varshney et al. 2002; Thiel et al. 2003; Gupta et al. 2003). The most frequent trinucleotide motif in *A. thaliana*, grape and *endophytes* is AAG (28.3% - 42.1%). Abundance of CCG repeats in monocot genomes may be due to their increased GC content (Morgante et al. 2002). The AAT motifs were the least common (<1%) in monocot species and in other species ranging from 17.4% in *S. cerevisiae* to 0 in primates (Cordeiro et al. 2002; Varshney et al. 2002). This may be explained by the fact that TAA-based variants code for stop codons that have a direct effect in protein synthesis in eukaryotes.

Other types of motifs (tetra-, penta- and hexa-nucleotides) are less studied. However, they can sometime be very frequent such as tetranucleotide repeats which exceed trinucleotide repeats in vertebrate introns and intergenic regions. Poly (A/T) motifs are the most abundant especially in plants but tetranucleotides are rare in ESTs (Toth et al. 2000; Gupta et al. 2004). Similarly the most frequent pentanucleotide motifs are A/T rich in vertebrates but are under-represented in coding regions (Toth et al. 2000). Hexanucleotide repeats constitute the second most frequent type after trinucleotide repeats in exons (Toth et al.

2000), whereas they are less frequent (<1%) in the whole genome of plants (Varshney et al. 2002; Cardle L. 2000), a dominance of (A+T)-rich repeats in introns and intergenic regions being observed in most of the species (Toth et al. 2000).

2.3.4 Number of repeats

The number of repeats is characteristic of an allelic variant at a given locus. This number depends on the type and of the size of the motif. A minimum number of six repeats is generally considered as appropriate for dinucleotides (Tautz 1993). In rice genome 72% of SSRs longer than 30 bp were of the dinucleotide type. In human, most of dinucleotide repeats had between 12 and 15 units (Weber et al. 1990). In general, g-SSRs have more repeats than EST-SSRs. For example, the mean number of units for rice g-SSRs is 16.5 while the mean number for EST-SSRs is 15.3 indicating that longer SSRs are mainly located in non coding regions (La Rota et al. 2005).

Similarly, in cereal ESTs, the frequency of SSRs decreases with increasing repeat length for all the species and every class of SSRs. In maize, the six repeat unit SSRs represent 56.9% of the total number of dinucleotide repeats SSRs and among the trinucleotide repeat SSRs, five repeat units share as much as 64.5% of total class. If all EST-SSRs of different types are classified into categories of <10 and >10 repeat units, the category of >10 repeats contributes only as much as 25% to the total number of SSRs. In the tetra- to hexanucleotide repeats SSR classes, all the EST-SSRs (100%) fall into the category of <10 repeat units (Varshney et al 2002).

2.4 SSRs within cereals

During the last decade, SSR markers were developed and investigated in a large number of plants including major cereal species such as barley (Thiel et al. 2003), maize (Chin 1996; Yu et al. 2001), oats (Li et al. 2000), rice (Gao et al. 2003), rye (Saal and Wricke, 1999), Sorghum (Bhattramakki et al. 2000) and wheat (Röder et al. 1998; Varshney et al. 2000; Guyomarc'h et al. 2002a, b; Gupta et al. 2002). In the majority of these, the two most common SSRs, whose density in the genome were determined while screening genomic libraries included GA and GT. The density of GA (38% to 59%) and GT (20% to 34%) in these different species ranged from one SSR every 212 kb to 704 kb (Varshney et al. 2002). Estimates of the total number of SSRs at the genome level were also performed in several

crops. The frequencies per haploid genome were one SSR each 3.6 X 10^4 bp for (GA)n and for 2.3 X 10^4 bp for (GT)n in bread wheat (Röder et al. 1995), and were estimated to be one SSR each 1.36 X 10^3 bp for (GA)n and for 1.23 X 10^3 bp for (GT)n in rice (Panaud et al. 1995).

In a recent survey of EST and genomic sequences, densities of SSRs were higher in the coding regions compared to non-coding regions of the genomes (Morgante et al. 2002). In EST sequences of several cereal species, it was estimated that the frequency ranged from one SSR every 3.9 kb in rice to one SSR every 7.5 kb in maize (Varshney et al. 2002). In wheat, the same authors mentioned one SSR every 6.2 kb while Gupta et al. (2003) reported one SSR each 9.2 kb.

Overall, an average of one SSR every 6-7 kb seems to be a good estimate for SSR frequency in the whole genome of plants (Cardle et al. 2000).

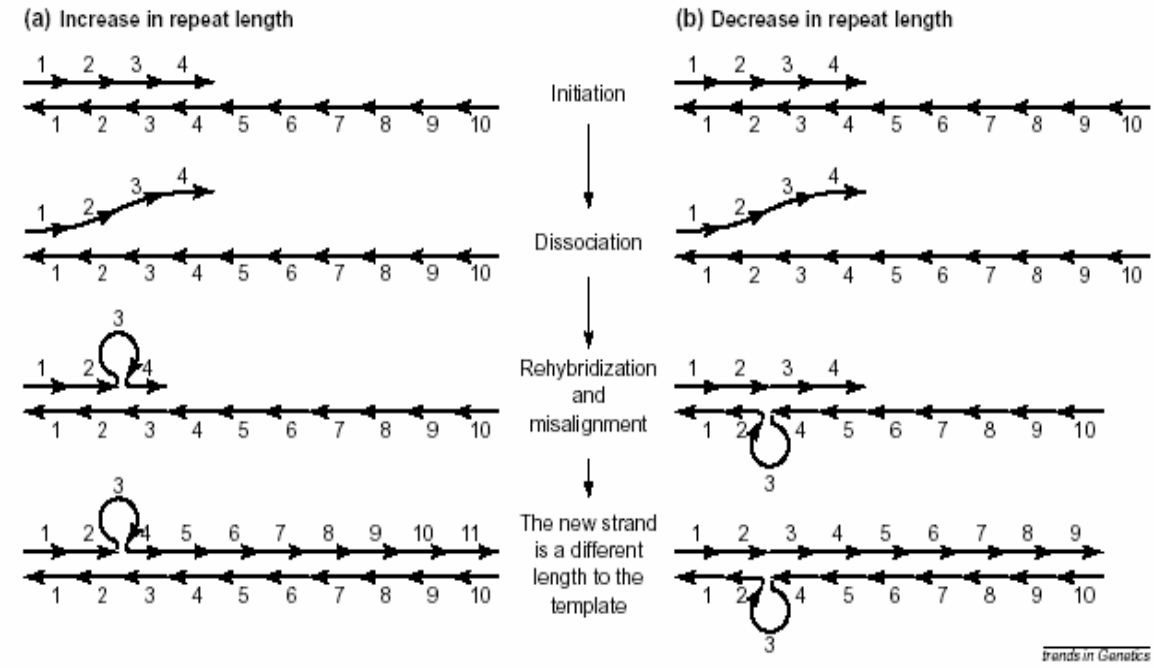
2.5Level of polymorphism of SSRs

2.5.1 Definition of SSR polymorphism

SSR polymorphism can be defined as the difference in the number of DNA repeats at a given locus, and can be easily analyzed through electrophoresis after PCR amplification. The level of polymorphism is generally evaluated through the Polymorphism Information Content (PIC) value (Nei et al. 1973) or diversity index (Akagi et al. 1998). However, it must be noticed that these values mostly depend on the sample studied (size and variability) and just allow comparison within the samples but not among different studies.

The sequences flanking specific SSR loci in a genome are supposed to be highly conserved within a species, but also in some cases across species, within a genus and rarely across related genera. These conserved sequences are therefore used to design primers for individual SSR loci. In human and various animals, SSRs have high mutation rates, ranging from 10^{-3} to 10^{-6}, which is superior to that of other regions in the genome (Bachtrog et al. 1999). In plants, the mutation rate at microsatellite loci is even higher than in animals, with the range of 10^{-2} to 10^{-3} in chickpea (*Cicer arietinum*, Udupa et al. 2001), or 10^{-3} to 10^{-4} in durum wheat (*Triticum turgidum*, Thuillet et al. 2002). SSR mutation rates are influenced by several factors, including motif type, perfection and number of repeats, among which the repeat number was best characterized (Schlötterer et al. 1998; Thuillet et al. 2004). In a variety of organisms, it was demonstrated that SSR mutation rates are

Figure 1-8 Model of SSR mutation by replication slippage (slipped-stand mispairing)



Repeat units are denoted by arrows. Numbers refer to the repeat unit number within each strand. (a) When the repetitive region is being synthesized the two strands can dissociate. Sometimes the strands are misaligned upon reassociation; that is, the nascent strand might incorrectly realign with repeat units downstream on the template strand. In such cases, a loop is formed on the nascent strand, and when synthesis of the new strand is initiated again it will become one repeat unit longer than the template strand. (b) As in (a), but the incorrect alignment occurs upstream on the template strand, and the new strand will therefore become one repeat unit shorter than the template strand.

positively correlated with repeat numbers and that the most polymorphic SSRs are the longest ones (Sia et al. 1997; Schlötterer et al. 1998; Thuillet et al. 2004). Weber (1990) reported that SSRs with 12 or fewer repeats were monomorphic in human (PIC = 0) and that the PIC value increased simultaneously to the average number of repeats, especially in the range of about 11 – 17 repeats.

2.5.2 Mutational mechanisms of SSRs

Two hypotheses are currently admitted to explain the variability of SSRs. The first one involves unequal crossing over between sister chromatides either during mitosis or after homologous pairing of chromosomes at meiosis (Wolf et al. 1989; Harding et al. 1992). However, they are most likely thought to accumulate by DNA slippage (Wells et al. 1965; Streisinger et al. 1966; Morgan et al. 1974; Efstratiadis et al. 1980; Drake et al. 1983; Levinson and Gutman 1987) and mispairing during replication and recombination or extension of single-strand ends (Tautz and Rentz 1984; Dover and Tautz 1986; Jeffreys et al. 1985; Wells et al. 1965).

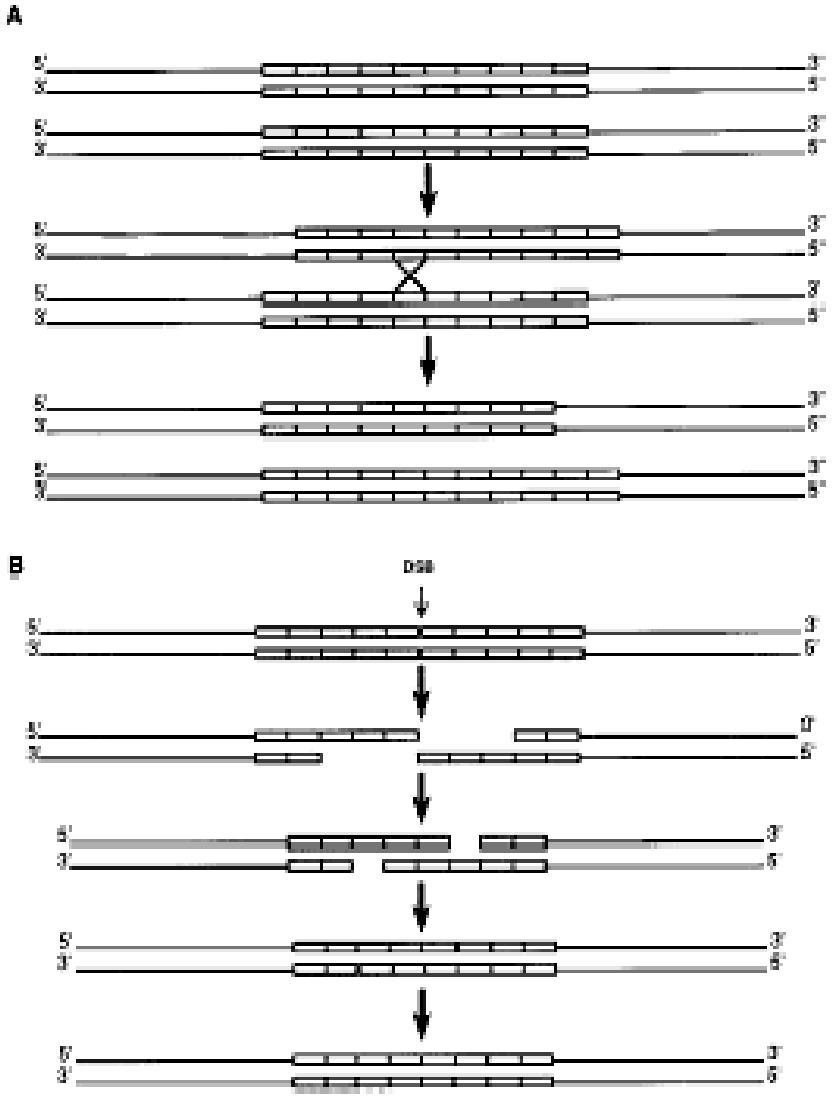
2.5.2.1 Replication slippage

A model for SSR mutation based on replication slippage was formulated by Levinson and Gutman (1987). Replication slippage or slipped strand mispairing refers to the out-of-register alignment of the two DNA strands following dissociation at the time when the DNA polymerase traverses the repetitive region. This slippage implies the formation of a single-strand loop during DNA synthesis and addition (or suppression depending on the strand concerned with the loop) of a motif. If the most 3’ repeat unit of the nascent strand re-hybridizes with a complementary repeat unit downstream along the template strand, a loop will be formed in the nascent strand and the new sequence will become longer than the template sequence upon elongation (Fig1-8a). On the contrary, if the incorrect alignment occurs upstream along the template strand, the new strand will become shorter than the template sequence (Fig1-8b). Most of these primary mutations are corrected by the mismatch repaired system, and only the small fraction that was not repaired ends up as microsatellite mutation events (Schlotterer and Tautz 1992).

2.5.2.2 Unequal recombination

Recombination could potentially change the SSR length by unequal crossing over or by gene conversion, which introduce mutation in the satellite sequence. In the version of the

Figure 1-9. Model of SSR mutation by recombination



The open rectangles represent individual repeat units. (A) Homologous recombination between misaligned arrays of 10 repeats results in one DNA molecule with a repeated tract one repeat unit longer than the original tract and one molecule that is one repeat unit shorter than the original tract. (B) A double strand break occurs within the repeated tract. This break is followed by exonuclease degradation that exposes complementary single-stranded regions. Hybridization of these regions followed by DNA synthesis and religation results in a reduction in repeat units. Alternatively, the broken ends could invade an array on another DNA molecule leading to an expansion in the tract.

recombination model shown in Fig1-9A, simple repeats located on different DNA molecules pair in a misaligned configuration and a crossover occurs, resulting in arrays with reciprocal additions and deletions. Other recombination models in which the additions or deletions are not reciprocal are also possible (Fig1-9B). There is little evidence that recombination would also contribute to microsatellite mutation. Genomic SSR distributions are associated with sites of recombination, most probably as a consequence of repetitive sequences being involved in recombination rather than being a consequence of it (Treco and Arnheim 1986; Majewski and Ott 2000)

2.5.2.3 Interaction between replication slippage and recombination

Other possible SSR mutation model was proposed by Li et al. (2002). When studying SSR diversity in wild emmer wheat, they found a strong interaction between mean repeat length and SSR locus distance from centromere. This interaction has an effect on the number of alleles and variance in repeat size at SSR loci. SSR mutation might thus be the result from the possible influence of replication slippage during recombination-dependent DNA repair. In fact, strand exchange between two homologous chromosomes should create a four-stranded configuration, called a Holliday structure, associated with mismatched (heteroduplex) DNA region. These regions undergo replication-dependent correction. Hence, a slippage mechanism may also work in recombination tracts involving SSR arrays (Gendral et al. 2000; Li et al 2002)

2.5.3 Polymorphism of g-SSRs

Numerous studies demonstrated that g-SSRs show a high level of polymorphism compared to other types of molecular markers. Kölliker et al. (2001) reported that SSR markers developed from white clover were highly polymorphic, 88% detecting polymorphism across seven genotypes with an average allele number of 4.8. In maize, PIC values ranged between 0.06 and 0.91 with a mean of 0.62 (Smith et al. 1997) which was similar to what is mentioned for RFLPs (Senior and Heun 1993). In barley, it was demonstrated that even if SSRs show lower diversity index compared to AFLP (0.521 and 0.937 respectively), they reveal a high number of allele at each locus making them very effective to study genetic relationships (Russell et al. 1997). In cultivated spelt wheat, Bertin et al. (2001) found that the mean PIC value was 0.64 while in common wheat; it was assessed to be 0.71. Even if lower PIC value (0.57) are also reported for common wheat (Stachel et al. 2000), this is twice higher than what is revealed by RFLPs (Chao et al. 1989; Cadalen et al.

1997).

2.5.4 Polymorphism of EST-SSRs

Assessments of polymorphism of EST-SSRs have been carried out in rice (Cho et al. 2000), grape (Scott et al. 2000), sugarcane (Grivet et al. 2003), tomato (Areshchenkova and Ganal, 2002), Alpine Lady-fern (Woodhead et al. 2003), pasture grass endophytes (Jong et al. 2002), barley (Thiel et al. 2003) and rye (Hackauf and Wehling 2002). In common wheat, EST-SSRs give better profiles compared to the g-SSRs (Holton et al. 2002) but the polymorphism level among EST-SSRs ranged from 7% to 55% (Holton et al. 2002; Eujayl et al. 2002; Gupta et al. 2003; Leigh et al. 2003; Nicot et al. 2004) which was lower compared to g-SSRs (61%, Sourdille et al. 2001; 100% Prasad et al. 2000). However, despite the fact that EST-SSRs show less polymorphism than g-SSRs, they are still informative for assessing genetic relationships (Eujayl et al. 2001, 2002; Gupta et al. 2003).

2.6 Role and function of the SSRs

2.6.1 DNA conformation

Alternations of purins and pyrimidins such as in (CG)n or (CA)n microsatellites favour the formation of Z-DNA (Bull et al. 1999). Z-DNA formed a double left-turned helix, which is more condensed compare to B-DNA because it contains 12 bp at each turn instead of 10.4 bp (for reviews see Loridon et al. 1998). Z-DNA is involved in recombination, gene expression regulation during transcription. On the contrary, homopurin and homopyrimidin sequences form H-DNA. Negative super-coiling of the DNA allows the formation of a triple helix resulting from the addition of a third DNA strand within the large groove of the double helix (Loridon et al. 1998). Other microsatellites may confer a hairpin loop structure and can thus play a role in the replication, the transcription, the translation and the interactions between DNA and proteins.

2.6.2 Promoter regulation

Microsatellites are frequently associated with regulatory elements (Levinson and Gutman 1987; Barrier et al. 2000). Hot spots for microsatellite formation within development homeotic genes such as the *antennapedia* and *dorsal* genes in Drosophila and *apetala* gene involved in floral regulation in plants can be identified (Barrier et al. 2000). Also, nuclear proteins specifically bind to repeated sequences such as (CCG)n which are frequently

encountered in the 5'URTs of the genes and which have thus an effect on mRNA transcription (Debrauwere et al. 1997).

2.6.3 Meiosis and mitosis

Microsatellites may also be involved in chromosome pairing at meiosis (Samadi et al. 1998) and in the centromere function (Levinson and Gutman 1987). It was also demonstrated that the tetranucleotide repeats (GACA)n were tightly linked to the nucleoli organizer in primates (Arens et al. 1995). Moreover, (GT)n SSRs have no effect on simple crossing over but increase multiple crossing over (Gendrel et al. 2000). The microsatellites inhibit Strand exchanges, which are promoted by RecA.

2.6.4 Coding function

Most of the microsatellites are distributed in euchromatin in coding as well non coding regions. Trinucleotide repeats locating in ORFs are coding for tandemly repeated amino acids that interfere with the protein function (Richard and Dujon, 1997). They can thus form proteic domains that are enriched in specific amino acids (Gortner et al, 1996). The most frequent codons are those coding for glutamine, asparagine and glycin.

In wheat, five microsatellites were initially identified in genes (Devos et al. 1995). Similarly, four genes bearing a microsatellite were reported in barley (Saghai-Maroof et al. 1994) and 27 in rice (Cho et al. 2000). With the rapid increase of ESTs in the databases (http://www.ncbi.nlm.nih.gov/dbEST), it is now well known that about 10% of the genes contain a microsatellite motif (Morgante et al. 2002, Nicot et al. 2004).

2.7 Application of SSRs to plant genetic studies

2.7.1 Genetic mapping

Breeding and genomics research efforts can make use of genetic maps to facilitate plant selection and to position BAC clones along physical maps of the genomes. In both cases, high throughput accurate marker technology is essential to avoid errors in genotyping and to enable processing of hundred or thousands of DNA samples in a short time. Microsatellites offer an easy tool and an abundant source of genetic markers. Consequently, SSR linkage maps have become available for a variety of plant genomes including rice (Wu and Tanksley 1993), barley (Saghai-Maroof et al. 1994), maize (Senior and Heun 1993) and wheat (Röder et al 1998; Somers et al. 2004). All these maps were proven to be

Tab1-3: Genome mapping using EST-SSR markers

Plant species	Number of genic SSR loci mapped	Mapping population used	Refs
Barley	185	3 DHs[a] (Igri×Franka, Steptoe×Morex, OWBDom×OWBRec)	Thiel et al.2003
	39	F_2s (Lerche×BGR41936), DHs (Igri×Franka), wheat－barley addition lines	Pillen et al. 2000
Cotton	111	BC_1[b] lines (TM1×Hai7124)×TM1)	Han et al. 2004
Kiwifruit	138	Intraspecific cross	Fraser et al. 2004
Raspberry	8	Full-sib family (Glen Moy×Latham)	Graham et al. 2004
Rice	91	DHs (IR64×Azucena), RILs[c] (Milyang 23×Gihobyeo, Lemont×Teqing, BS125×WLOGenome mapping using EST-SSRs 2)	Temnykh et al. 2000
Rye	39	4 mapping populations derived from reciprocal crosses (P87×P105, N6×N2, N7×N2, N7×N6)	Khlestkina et al. 2004
Ryegrass	91	Three-generation population (Floregon×Manhattan)	Warnke et al. 2004
Tall fescue (*Festuca* spp.)	91	Pseudo-test cross-population (HD28－56×R43－64)	Saha et al. 2004
Wheat	149	RILs (W7984×Opata85)	Yu et al.2004
	126	RILs (W7984×Opata85)	Nicot et al. 2004
	101	RILs (W7984×Opata85, Wenmai 6×Shanhongmai), DHs (Lumai14×Hanxuan 10)	Gao et al. 2004
White clover	449	Pseudotest cross-population (6525/5×364/7)	Barrett et al. 2004

[a] Abbreviations: BC_1, backcross population; DHs, doubled haploids; RILs, recombinant inbred lines.

useful for QTL detection of numerous traits of agronomic interest and positional cloning of genes underlying these QTLs. Recently, the EST-SSR loci have been integrated for a wide range of species (Tab 1-3). Contrary to g-SSRs, EST-SSRs were not clustered around the centromere but, as expected, were concentrated in gene-rich regions (Thiel et al. 2003; Yu et al. 2004; Gao et al 2004). It is believed that the distribution of EST-SSRs in the genetic maps mirrors the distribution of genes along the genetic map. Moreover, EST-SSRs may affect the genes' function and thus constitute gene-targeted markers with the potential of representing functional markers in those cases where polymorphisms in the repeat motifs affect the function of the gene in which they reside (Anderson et al. 2003). They can contribute to 'direct allele selection', when completely associated or even responsible for a targeted trait (Sorrells et al. 1997). Recently, for example, a *Dof* homolog (*DAG1*) gene that showed a strong effect on seed germination in *Arabidopsis* has been mapped on chromosome 1B of wheat by using wheat EST-SSR primers (Gao et al. 2004). Similarly, Yu et al. (2004) identified two EST-SSR markers linked to the photoperiod response gene (*ppd*) in wheat. In both cases, these may constitute candidate genes or this may help in positional cloning of the gene because of the likely existence of gene rich islands where several genes are clustered. Finally, mapping EST-SSRs can facilitate genome alignment across distantly related species (Yu et al. 2004; Varshney et al 2005) as classical RFLP markers are currently less used for that.

2.7.2 Genetic diversity

Characterization of genetic variation within natural populations and among breeding lines is crucial for effective conservation and exploitation of genetic resources for crop improvement programs. SSRs have proven to be useful for assessment of genetic variation in germplasm collections of various species (Brown et al. 1996; Struss et al. 1998; Djè et al. 2000; Ghebru et al. 2002; Matsuoka et al. 2002; Ni et al. 2002; Sjakste et al. 2002; Chebotar et al. 2003; Russell et al. 2003; Yu et al. 2003; Zhou et al, 2003; Tarter et al 2004; Casa et al. 2005; Clerc et al. 2005; Menkir et al. 2005; Mohammadi et al. 2005). However most of the markers derived from genomic DNA assay polymorphisms potentially carried by non-coding regions of the genome and are poorly conserved among species (Brown et al. 2001). Therefore, these markers overestimate the "useful" genetic diversity available in the gene pool. In contrast, EST-SSRs generally assay polymorphism potentially carried by the coding regions of the genome and can detect the "true functional genetic diversity"

(Maestri et al. 2002; Yamanaka et al. 2003; Gupta et al. 2003). In recent years, EST-SSRs have also been used for diversity estimations in several plant systems including durum wheat (Eujayl et al. 2001), *Pisum* sp. (Burstin et al. 2001), barley (Thiel et al. 2003; Holton et al. *2002*), bread wheat (Holton et al. 2002; Gupta et al. 2003; Gao et al. 2003), rye (Hackauf and Wehling 2002). In comparison to genomic SSRs, EST-SSRs revealed less polymorphism in germplasm characterization and genetic diversity studies (Scott et al. 2000; Thiel et al. 2003; Cho et al. 2000, Russell et al 2004). Therefore, evaluation of germplasm with EST-SSRs enhance the role of genetic markers by assaying the variation in transcribed regions, although there is a higher probability of bias owing to selection. Thereby, the genetic diversity presented by combining two types of markers might be representative of the entire genome. In addition, EST-SSRs also provide opportunities to examine functional diversity in relation to adaptive variation (Eujayl et al 2001; Russell et al 2004).

2.7.3 Comparative mapping

Numerous studies demonstrated that g-SSRs are frequently locus specific without corresponding homoeoloci on the other related genomes (Bryan et al. 1997; Stephenson et al. 1998; Röder et al. 1998). Thus, they appeared to have a limited transferability to related species (Sourdille et al. 2001; Varshney et al. 2000) suggesting that they are of limited utility, when used for comparative mapping. On the contrary, numerous studies revealed that EST-SSRs showed a high level of transferability to close and wild relatives because they are derived from conserved coding regions (Holton et al. 2002; Gupta et al. 2003; Eujayl et al. 2001, 2002; Bandopadhyay et al. 2004). Recently, the potential use of EST-SSRs developed for barley and wheat has been demonstrated for comparative mapping in wheat, rye and rice (Yu et al. 2004; Varshney et al 2005). These studies suggested that EST-SSR markers could be used in related plant species for which little information is available on SSRs or ESTs. In addition, the EST-SSRs are good candidates for the development of conserved orthologous markers for genetic analysis and breeding of different species. For example, a set of 12 barley EST-SSR markers was identified that showed significant homology with the ESTs of four monocotyledonous species (wheat, maize, sorghum and rice) and two dicotyledonous species (*Arabidopsis* and *Medicago*) and could potentially be used across these species (Varshney et al 2005).

Similarly, orthology can only be determined by comparing both similarity of amplicon

sequences and genome location across species (Yu et al. 2004; Varshney et al 2005). For example, Saha et al. (2004) who sequenced the products of one EST-SSR primer pair for three fescue species, ryegrass, rice and wheat reported that sequences were >85% similar. Sequence-based comparison of mapped barley EST-SSRs with genetically and/or physically mapped markers in wheat, rye and rice revealed several markers that showed an orthologous relationship between examined cereal species (Varshney et al 2005). Comparison of genome locations of polymorphic EST-SSR markers mapped in both wheat and rice also confirmed previously known genome relationships with most of the markers examined (Yu et al. 2004). However, the assessment of colinearity was complicated by the detection of multiple polymorphic loci in either wheat or rice for 85% of the primer pairs suggesting sequence divergence or differential gene duplication.

2.7.4 Phylogenetic studies

Genomic SSRs were extensively explored in plant evolutionary studies because of their high polymorphism level, their co-dominant inheritance, and their reproducibility (Plaschke et al. 1995*;* Prasad et al. 2000; Manifesto et al. 2001; Ben Amer et al. 2001; Leisova and Ovesna 2001; Zhang et al. 2002). However, interspecific phylogenetic studies in using SSRs were often restricted because of the limited transferability of genomic SSRs to related species (Lelley et al. 2000) and the high mutation rate. Because being highly transferable to distantly related species, EST-SSRs are thought to be more suitable for this purpose. EST-SSRs had been used to investigate their potential in providing useful information for phylogeny (Rossetto et al. 2002). Three EST-derived microsatellite loci from *Vitis vinifera* were amplified and sequenced across eight species of *Vitaceae* from four different genera. Phylogenetic analysis of the microsatellite's flanking regions produced informative results in congruence with previous studies. Based on EST-SSRs allelic polymorphism among 18 species of *Triticum-Aegilops* complex using 64 common wheat EST-SSRs, Bandopadhyay et al. (2004) were able to construct a dendrogram separating the diploid and tetraploid species.

2.7.5 Marker Assisted Selection (MAS)

Application of MAS has been shown to increase selection efficiency, particularly for traits with low heritability (Bernardo et al. 2001). It will be increasingly applied to accelerate selection of traits that are difficult to manage via phenotype, owing to low penetrance and/or complex inheritance. It will also serve to maintain recessive alleles in backcrossing

pedigrees, to pyramid disease-resistance genes and to aid in the choice of parents in crossing programs, to ensure minimal levels of duplication of alleles across sets of genes targeted for selection, and to promote fixation.

Recently, increasing numbers of agronomically significant genes have been tagged with linked SSR assays (Huang et al. 2000; Raupp et al. 2001; Bariana et al. 2001). Most of these are resistance genes because single-gene control of this class of character is widespread. For example, Hurtado et al. (2002) demonstrated that SSRs tightly linked to sharka resistance facilitated MAS in breeding for resistance in apricot.

However, most traits of agronomical interest are under polygenic control such as the resistance to *fusarium* head blight (FHB), which is a major objective for many private as well as public laboratories in the world. For practical reasons, FHB is a difficult disease to handle by conventional pathology testing, and genetic analysis of the primary sources of resistance has shown that a significant proportion of the effect can be attributed to three QTL, mapping to different chromosomes, with each of the relevant genomic sites tagged with the genomic SSR locus (Anderson et al. 2001). Similarly in rice, numerous g-SSRs were found to be closely associated with low glutelin content and their effectiveness in MAS breeding was illustrated (Wang et al. 2002). EST-SSRs are more likely to exhibit perfect marker-trait association, provided that the gene containing SSR is the QTL itself. This perfect marker-trait association must be assessed by fine association mapping (linkage disequilibrium = LD) prior to MAS. Through marker-trait association, these so-called “perfect markers” can be developed for a variety of traits in a large number of plant systems, once candidate genes have been found.

3 Organization of genetic resources

3.1History

Agricultural practices prior to the eighteenth century were completely dependent on crop landraces and mixtures of these landraces. The Industrial Revolution was followed by a simultaneous population explosion that transformed the subsistence nature of agriculture and its farming systems forever. In the mid nineteenth century, Mendel (and other pioneering plant geneticists such as Vilmorin) provided knowledge of plant genetics that made it possible to dramatically increase the production potential of agriculture. Undeniably, the wonders of crop improvement have resulted in the erosion of genetic diversity of many crops in farmers' fields, including wheat, due to the replacement of many, heterogenous landraces and farmers' old cultivars by fewer modern high-yielding varieties.

3.2Definition and classification

Genetic resources are genetic material with an effective or potential value, which encompass the diversity of genetic material both in traditional varieties and modern cultivars, as well as crop wild relatives and other wild plant species. Besides, they are fundamental to sustain global production now and especially in the future.

Genetic resources include a wide range of genetic diversity that is critical to enhance and maintain the yield potential and to provide new sources of resistance and tolerance to biotic as well as abiotic stresses. Modern high-yielding cultivars are an assembly of genes or gene-combinations pyramided by breeders using, in most cases, well-adapted cultivars from their regions. International agriculture research has enormously expanded the availability of widely adapted germplasm that is genetically diverse (i.e. descended from more sources). Genetic resources are also a reservoir of genetic adaptability to buffer against potentially harmful environmental and economic changes. However, introgression of additional variation found in genetic resources is necessary to increase yield stability and further improvement.

Genetic resources were categorized by Frankel (1977) and the Food and Agriculture Organization of the United Nations Commission on Plant Genetic Resources (FAO, 1983), though this categorization is not followed by all centers involved in genetic resource conservation and utilization. These categories are:

- modern cultivars in current use;
- obsolete cultivars, often the elite cultivars of the past and often found in the pedigrees of modern cultivars;
- landraces;
- wild relatives of crop species in the *Triticeae* tribe;
- genetic and cytogenetic stocks;
- breeding lines.

These genetic resources constitute the gene pool available for breeders and other scientists, and in the *Triticeae* tribe several pools are recognized (Bothmer *et al*., 1992). The primary gene pool consists of the biological species, including cultivated, wild and weedy forms of crop species. Gene transfer in the primary gene pool is considered to be easy. In the secondary gene pool are the coenospecies from which gene transfer is possible but difficult, while the tertiary gene pool is composed of species from which gene transfer is very difficult or almost impossible.

3.3Collections

3.3.1 Landraces

Landraces refers to the particular kind of old seed strains and varieties that are farmer-selected in areas where local subsistence agriculture has long prevailed. Landraces are highly adapted to specific locales or groups. The term is usually applied to varieties of wheat, corn, squash, and beans that were domesticated by native farmers, and further modified by native and also immigrant farmers.

Nicolai Vavilov initiated works in the context of landraces in 1926. He firstly engaged in studying the geographical situation of landraces and investigated the origins of these landraces. His studies approved the concepts of “the origin center” and “the diversity center” for landraces, referring to some cultivars in certain region showing a very strong diversity, which made it feasible to well understand their diversity and to well collect genetic resources.

In order to manage these genetic resources, CGIAR (Consultative Group on International Agricultural Research) was created in 1971. CGIAR is a strategic alliance of countries,

Tab 1-4: Number of accession available in wheat collections around the world (Source: Information collared from IBPGR, 1990)

Type of wheat	Number of accessions
Hexaploid	266 589
Tetraploid	78 726
Diploid	11 314
Unspecified *Triticum*	252 530
Aegilops ssp	17 748
Triticale	23 659
Total	650 566

international and regional organizations, and private foundations supporting 15 international agricultural Centers (list in annex 1), which work with national agricultural research systems and civil society organizations including the private sector. In CGIAR's gene banks, about 640 000 accessions of *Triticum* ssp., *Aegilops* ssp. and X *Triticosecale* can be found for global wheat genetic resources (Tab. 1-4). In order to collect these genetic resources, many missions were carried out with regard to *Triticum* genus. For example, a collection was completed in Albanie Mountain in 1995 (Hammer et al. 2000). One hundred and twenty accessions of landraces and wild species were collected, including some traditional species, such as *Triticum monococcum*. In the same year, another task was also performed in southern Sardinia and 80 samples were gathered in 22 different sites, principally with focus on cereals (including *Triticum* genus), leguminous and vegetables. Some genetic erosion was observed for these accessions.

3.3.2 Wild species

Wild species refers to plants growing in the wild that have not been subject to domesticating to alter them from their native state.

In order to conserve wild species, an organization – Botanic Gardens Conservation International (BGCI) – was founded in United Kingdom in 1987. To date, more than 500 members from 112 different countries work in BGCI to efficiently conserve these genetic resources. There are botanic gardens and arboreta in 148 countries worldwide and they maintain more than 4 million living plant collections. Amongst their collections are representatives of more than 80,000 species, almost one third of the known vascular plant species of the world. There are a total of 142 million herbarium specimens in botanic garden herbaria and 6.13 million accessions in their living collections. Over 500 botanic gardens occur in Western Europe, more than 350 in North America and over 200 in East and Southeast Asia, of which the majority are in China. Most of the southern Asian botanic gardens are found in India. There are seven research centers, distributed in USA, Canada, China, Brazil, Japan, India and Russia. The main objectives of this organization include a) Understanding and documenting plant diversity; 2) Conserving plant diversity; 3) Using it sustainably; 4) Promoting education and awareness about plant diversity; 5) Building capacity for its conservation.

3.4Evaluation of genetic resources

Evaluation should be a major activity of germplasm banks to identify useful genetic variation and make it available to breeders. There are many methods for depicting and evaluating diversity of genetic resources (Lefort-Buson et al. 1988). Several descriptive levels exist: agronomical level, technological level, biochemical level and molecular level. Information from each level is specific, and shows some advantages and drawbacks. In the following part, these methods will be presented one by one, and compared. Because numerous studies on genetic diversity have been made on plant species, we will focus our presentation on grass species only and mainly on wheat.

3.4.1 Agronomical and morphological data

The morphological traits were the first descriptive markers used. They are simple and easy to obtain. The information derived from morphological character was an important characteristic for classification and systematics of the studied material. With regard to grass species, many researches based on morphological data were performed. In 2000, Nieto-Lopez identified significant intra- and inter-population diversity within a collection of wild populations in *Elymus* and *Thinopyrum* genus by using 22 morphological and agronomical traits. Several similar studies were achieved for *Poaceae* species, such as maize (Malosetti et al, 2001) and wheat (Jardat, 2001; Brandolini et al, 2002; DeLacy et al, 2000, Grenier et al, 2001). For wheat landraces, 465 individual spikes of bread wheat were collected from 24 sites in three states of Mexico in 1992. They were examined for 15 morphological, agronomic and grain quality attributes as part of the routine regeneration process conducted by the CIMMYT Wheat Genetic Resources Program in unreplicated hill plots in a screen house. A pattern analysis (combined use of classification and ordination methods) of the data showed a good description of the accessions and the collection sites, suggesting that the analysis for economically useful attributes could provide relevant information for users and the germplasm curators (DeLacy et al. 2000).

3.4.2 Isozymes data

Isozymes were first described by Hunter and Markert (1957) who defined them as different variants of the same enzyme having identical functions and present in the same individual. This definition encompasses (1) enzyme variants that are the product of different genes and thus represent different loci (described as isozymes) and (2) enzymes that are the product

of different alleles of the same gene (described as allozymes). Isozymes constitute a powerful tool for genetic diversity study within and among population of plants and animals. Isozymes may be almost identical in function but may differ in other ways. In particular, amino acid substitutions that change the electric charge of the enzyme (such as replacing aspartic acid with glutamic acid) are simple to identify by gel electrophoresis, and this forms the basis for the use of isozymes as molecular markers.

Isozymes have been the most widely used molecular markers to identify genetic variation within and between populations. Genetic diversity in 79 European accessions of the Barley Core Collections was surveyed using isozyme electrophoresis. Results on a total of 26 alleles observed at ten isozyme loci demonstrated that 6-rowed barley contained larger diversity than 2-rowed barley and that winter type contained larger diversity than spring type (Liu et al. 2000). Also for barley, Kaneko et al. (2002) investigated the polymorphism and geographical distribution of β-amylase isozymes by isoelectric-focusing (IEF) analysis in a sample of world barley. The isozyme pattern of high thermostability type A and low thermostability type C varieties was restricted in isozyme type II. However, the isozyme pattern of the middle thermostability type B varieties was polymorphic.

Concerning wheat, a large amount of studies focused on wheat endosperm storage proteins: gliadins and glutenins, because they play an important role in dough properties and in bread making quality in various wheat varieties. Therefore, many researches were performed in order to survey genetic diversity of genes coding these proteins (Metakovski and Branlard 1998; Branlard et al. 2001; Ruiz 2002). For example, by using SDS - PAGE, the different alleles encoded at the 6 glutenin loci and at 3 ω-gliadin loci were identified from a set of 200 hexaploid wheat cultivars grown primarily in France (Branlard et al. 2003), and some associations were revealed due to pedigree relatedness between some French wheat cultivars. These studies allowed to better understanding genetic diversity of protein-coding genes, which can be useful for future genetic and technological wheat improvement.

3.4.3 NIRS data

With increases in the processing speed of PCs, and in the capabilities of the softwares, Near Infrared Spectroscopy (NIRS) data have been used to analyze and measure the concentration of a number of compounds simultaneously in more complex mixtures. For cereals, NIRS method has been used to investigate diversity in rice varieties (Krzanowski

et al. 1995) in order to identify differences between Basmati and other rices. Moreover, Roussel et al (2005) studied wheat components (proteins, minerals, fatty acids and carbohydrates) in a set of 539 French bread wheat accessions released and cultivated during the last two centuries in France. Results showed that whole NIRS spectra provided a useful tool for describing the global evolution of the chemical composition of the grain of French wheat.

3.4.4 Molecular data

3.4.4.1 RFLP

Restriction Fragment Length Polymorphism (RFLP) is a technique that uses restriction endonucleases (RE) to cut DNA at specific 4-6 bp recognition sites and resulting fragments are separated according to their molecular size using gel electrophoresis. Presence and absence of fragments resulting from changes in recognition sites are used identifying species or populations. Because RFLPs were the first molecular markers developed, they have been widely used for diversity analyses in numerous plant species. Only selected results on wheat will be presented here.

Genetic diversity in a set of 11 red and 11 white wheat lines from the Eastern U.S. soft wheat germplasm pool was measured using restriction fragment length polymorphism (RFLP) assay (Kim and Ward 1997). It revealed that the frequency of polymorphism in the Eastern U.S. soft white winter (SWW) wheat gene pool was much lower than that observed in the Eastern U.S. soft red winter (SRW) wheat gene pool. In another study, genetic diversities were investigated in landraces of *T. aestivum* from China (38) and Southwest Asia (55) by RFLP analysis (Ward et al. 1998). A total of 368 bands were found for 39 Chinese hexaploid wheat accessions with 63 RFLP probe-*Hin*dIII combinations. Results showed that the individual Chinese landrace wheat groups revealed less variation than those from Afghanistan, Iran, and Turkey. Also, a narrow genetic variability was revealed among 17 populations of wild emmer wheats sampled from South-eastern Turkey, by using eleven RFLP clones and 4 restriction enzymes (Tanyolac et al. 2003). All the published works demonstrated that RFLPs were an effective marker to study genetic diversity.

3.4.4.2 RAPD

Random Amplified Polymorphic DNA (RAPD) analysis described by Williams *et al.*

(1990) was a commonly used molecular marker in genetic diversity studies. The principle involved in generating RAPDs is that, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragments generated by PCR depend on the length and size of both the primer and the target genome. The assumption is made that a given DNA sequence (complementary to that of the primer) will occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR. These amplified products (of up to 3.0 kb) are usually separated on agarose gels and visualised by ethidium bromide staining. The use of a single 10-mer oligonucleotide promotes the generation of several discrete DNA products and these are considered to originate from different genetic loci. Polymorphisms result from mutations or rearrangements either at or between the primer binding sites and are detected as the presence or absence of a particular RAPD band. This means that RAPDs are dominant markers and therefore cannot be used to identify heterozygotes.

RAPDs have been largely exploited in genetic diversity researches. A set of 86 RAPD markers was used to characterize the USDA *Poa pratensis* collection (Johnson et al. 2002), and illustrating that RAPD was a very useful tool in species identification and diversity estimation within accessions. In order to study genetic diversity among Croatian wheat cultivars, 36 RAPD primers were screened and the 14 most polymorphic ones yielded 341 polymorphic bands. RAPD markers showed a high level of polymorphism among the cultivars examined and the breeding lines. (Maric et al. 2004).

3.4.4.3 AFLP

The AFLP (Amplified Fragment Length Polymorphism) technique was described by Vos et al. (1995) and is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique involves three steps: (i) restriction of the DNA and ligation of oligonucleotide adapters, (ii) selective amplification of sets of restriction fragments, and (iii) gel analysis of the amplified fragments. PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. The selective amplification is obtained by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extensions match the nucleotides flanking the restriction sites. Using this method, sets of restriction fragments may be visualized by PCR without knowledge of nucleotide

sequence.

AFLP has been wildly used in genetic diversity analysis. Genetic diversity was estimated within and between populations of perennial ryegrass (*Lolium perenne* L., Guthridge et al. 2001). Cluster analysis of AFLP data from bulked samples produced a phenogram showing relationships consistent with the results of individual analysis. These results suggested that AFLP profiling provides an important tool for the detection and quantification of genetic variation in perennial ryegrass. A study of the genetic variability in 94 genotypes from ten populations of wild barley, *Hordeum spontaneum* (C. Koch) Thell., originating from ten ecologically and geographically different locations in Israel, was performed (Turpeinen et al, 2004). It revealed that genetic diversity was larger within (69%) than among (31%) populations.

For wheat, Roy et al (2004) performed a comparison between AFLP-based genetic diversity with diversity based on SSR, SAMPL, or phenotypic traits in 55 elite and exotic bread wheat lines. Results showed that AFLP was superior for estimation of genetic diversity for landraces to other molecular markers. Moreover, a group of 54 synthetic hexaploid wheats derived from crosses between emmer wheat (*Triticum dicoccum*, source of the A and B genomes) and goat grass (*Aegilops tauschii*, D genome donor) were investigated for genetic diversity. Based on data from four AFLP primer pairs, dendrograms revealed clear grouping according to geographical origin for the *T. dicoccum* parents but no clear groups for the *Ae. tauschii* parents (Lage et al. 2003). This study also suggests that synthetic hexaploid wheats can be used to introduce new genetic diversity into the bread wheat gene pool. In 2002, a substantial amount of between- and within-cultivar genetic variation was detected in all the 13 registered modern Canadian durum wheat (*Triticum turgidum* L. ssp. durum (Desf.) Husn.) cultivars based on AFLP. Among the approximately 950 detected AFLP markers, only 89 (average of 8.9 polymorphic loci per primer pair) were polymorphic, 41 showing polymorphism between cultivars whereas the remaining 48 showed polymorphism within at least one cultivar. Otherwise, the level of genetic variation among individuals within a cultivar at the breeders' seed level was estimated based on an inter-haplotypic distance matrix derived from the AFLP data. Results show that the level of genetic variation within the most-developed cultivars is fairly substantial despite rigorous selection pressure aimed at cultivar purity in breeding programs (Soleimani et al. 2002).

3.4.4.4 Microsatellites

Numerous works were carried out in cereals like rice (Ni et al. 2002; Yu et al. 2003; Zhou et al, 2003), maize (Matsuoka et al. 2002; Tarter et al 2004, Clerc et al. 2005; Menkir et al. 2005), sorghum (Brown et al. 1996; Djè et al. 2000; Ghebru et al. 2002; Casa et al. 2005), barley (Struss et al. 1998; Sjakste et al. 2002; Russell et al. 2003), and rye (Chebotar et al. 2003). In the case of wheat, this type of marker was also largely used to study genetic diversity among European varieties (Röder et al, 2000), in European bread wheat varieties released from 1840 to 2000 (Roussel et al 2005), among European cultivated spelt (Bertin et al. 2001), among old and modern Siberian varieties (Khlestkina et al, 2004), in natural populations of wild emmer wheat, *Triticum dicoccoides*, in Israel (Fahima et al. 2002). Through these studies, we are able to better understand the diversity in these species, as well as their evolution.

3.4.4.5 Inter- Simple Sequence Repeat (ISSR)

Inter-simple sequence repeat or ISSR is an efficient DNA fingerprinting method that do not require prior knowledge of the nucleotide sequence to be analyzed. One single ISSR reaction can simultaneously identify multiple polymorphisms at various loci throughout the genome, depending on the amount of variation between the cultivars studied. ISSR techniques were firstly described by Zietkiewicz et al. (1994) and are used to detected polymorphisms for a short DNA sequence between two SSR in the genome, by designing primers from microsatellite regions. ISSR has been widely used for genetic diversity studies in barley (Tanyolac et al. 2003), *Lolium perenne* (Ghariani et al. 2003), rice (Bao et al. 2006). For example, Ghariani et al (2003) examined the genetic diversity in Tunisian perennial ryegrass (*Lolium perenne*) by using inter-simple sequence repeats (ISSR). One hundred and thrity six polymorphic ISSR markers (average of 12.6 polymorphic bands/primer) were used to estimate the genetic distance among eighteen accessions, to draw phylogenetic trees, and to provide evidence of a high degree of genetic diversity in Tunisian ryegrass.

3.4.4.6 Sequence Tagged Site (STS)

Sequence tagged site or STS is a short unique genomic sequence that is amplified by using allele-specific oligonucleotides as PCR primer. When mapped RFLP or gene sequence

information is available, it can be used to design PCR-based STS primers. To date, STS has been used to analyze the genetic diversity for several agricultural crops, such as in Hordeum (Chen et al. 2005). To assess the genetic diversity among China's cultivated barley, sequence tagged site (STS) marker analysis was carried out to characterize 109 morphologically distinctive accessions originating from five Chinese eco-geographical zones. Fourteen polymorphic STS markers representing at least one on each chromosome were chosen for the analysis. They revealed a total of 47 alleles, with an average of 3.36 alleles per locus (range 2–8). The result suggested that the STS diversity in different zones was quite different from the morphology diversity, and indicated that the STS variation was partitioned into 17% among the zone and 83% within the zone (Chen et al. 2005).

3.4.4.7 Single Nucleotide Polymorphisms (SNPs)

Single Nucleotide Polymorphism or SNP is a DNA sequence variation, occurring when a single nucleotide (adenine (A), thymine (T), cytosine (C) or guanine (G)) is altered in the genome (*i. e.* substituted, deleted or added). It is estimated that there is probably one SNP locus every 500 to 1,000 bp between two individuals randomly sampled in the same species. This large abundance suggests that SNPs markers can be useful for numerous genetic applications. For example, SNPs were approved as an effective mean of characterizing the range of DNA variation at a genomic scale in *Arabidopsis thaliana* and to build up a core collection (McKhann et al. 2004). Since SNPs appeared recently, only few studies used this method for genetic diversity estimation in cereals and they were focused on genes of interest. For example, in 2003, Yanagisawa et al. investigated a single nucleotide polymorphism (SNP) in the Wx-D1 gene of wheat by using a derived cleaved amplified polymorphic sequence (dCAPS) marker, and showed that the SNP in the Wx-D1 gene was responsible for the waxy character. Furthermore, this type of marker was also demonstrated to be a very useful tool to study linkage disequilibrium in common wheat (Ravel et al. 2005) as well as in barley (Potokina et al. 2006).

3.4.4.8 Expressed Sequence Tag (EST)

An Expressed Sequence Tag or EST is a short sub-sequence of a transcribed protein-coding or non-protein-coding DNA sequence. ESTs can be derived in PCR-based markers, which can detect length and sequence polymorphisms carried by the expressed regions of plant genomes. This involves the designing of primers separated by an

Tab 1-5: Comparison of different molecular markers

Markers	Advantages	Disadvantages
Morphological markers	Easy monitor	Affected by the enviroment Limited number of avaible markers Sometime difficult to score
Allozymes	Cheap Samlp being prepared easily Standard statistical procedure	Limited number of avaible markers Some loci show protein instability Potentially direct target of selection
RFLPs	Co-dominant inheritance Reproductibility Highly tansferable across distant relatives	Low infornation content Pollution of isotope Requirement of a huge quantity of DNA Impossible to automate
RAPDs	Cheap Relatively high information content Requirement neither DNA probe nor sequence information Technique quick and simple Requirement of small amounts of DNA Automation	Low reproducibility Dominant inheritance
AFLPs	High information content Reproductibility Genome-specific Automation	Expensive Dominant inheritance
SSRs	Abundant dispersion on the whole genome High information content Co-dominant inheritance Reproductibility Locus specificity for g-SSRs Highly tansferable across distant relatives for EST-SSRs Automation	High mutation rate Complex mutation behaviour
ISSRs	High information content Co-dominante Reproductibility No requirement of sequence information Technique quick and simple	High mutation rate

amplifiable EST segment, and use of these primers for PCR amplication of gDNA. The growing numbers of ESTs in databsaes has provided a valuable resource for EST markers. They have been successfully used in several species of *Pinus* (Harry et al. 1998) and *Picea* (Brown et al. 2001) to serve as links between the maps of different species. Moreover, several EST-derived molecular markers have been widely exploited for phylogenetic studies, such as EST-SNPs (McKhann et al. 2004) or EST-SSRs (Eujayl et al. 2001; Burstin et al. 2001; Thiel et al. 2003; Holton et al. 2002; Gupta et al. 2003; Gao et al. 2003; Hackauf and Wehling 2002).

3.4.4.9 Comparison between the different types of markers for diversity analyses

Advantages and drawbacks of the different types of markers are given in Tab. 1-5. It is well known that morphological data are the less reliable markers because of their strong interactions with environment. They are in a limited number, and sometime difficult to score. This is why molecular markers were primarily developed. Compared with other markers, isozymes are relatively inexpensive. Large samples can be processed with far less training and time per sample, and standard statistical procedures can be used for fine and broad scale genetic variation studies. However, their number is also limited (about 40) and only few alleles exist at each locus. In recent years, there has been an explosion in the number of different types of DNA markers available, which provide the same type of information as isozymes, but allow clearer resolution of genetic differences and which can be found in an almost unlimited number. In the past decades, RAPDs have been widely used because they require neither DNA probe nor sequence information. In addition, the procedure involves no blotting or hybridization steps leading therefore the technique quick, simple and efficient. RAPDs also require only small amounts of DNA (about 10 ng per reaction) and the procedure can be automated. However, RAPDs are known to be only poorly reliable and reproducible between the laboratories. Moreover, these markers are mainly dominants and heterozygotes cannot be detected. Concerning AFLPs, the method allows the specific co-amplification of a high number of fragments. Typically 50-100 fragments are amplified and detected on denaturing polyacrylamide gels. The AFLP technique provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity. However, like RAPDs, these are mostly dominant markers and only two alleles are observed at each locus (presence/absence of a band). RFLPs are mainly codominant markers which show a quite high level of polymorphism and several

STSs	Locus specificity Reproductibility Co-dominate	Requirement for sequence information
SNPs	Low mutation rate High abundance East to type New analytical approches are being developed at present	Substaintial rate heterogeneity among sites Expensive to isolate Low information content of single SNP
ESTs	Cheap Association with phenotypes	Low information content

alleles at each locus (mean ~3). In addition, they often detect homoeologous copies and can thus be used in comparative studies. However, they require a huge quantity of DNA (several µg) and are also impossible to automate. Thus, only few samples can be run simultaneously. SSRs exhibit all the advantages of the other markers. They are codominant, highly reproducible and polymorphic (twice more than RFLPs), they can be easily automated and are mainly genome-specific in wheat. However, due to this latter point, they cannot be used in comparative studies and in addition; they are very expensive to develop because of the high number of sequences that must be made prior to have a useful marker. Despite this, they constitute the marker of choice for genetic studies in wheat.

3.5 Aims of the thesis

3.5.1 Scientific question

As previously described, the *Triticeae* species show a high genetic variability that can be useful for wheat genetic improvement. They can constitute a huge reservoir of new alleles for biotic and abiotic resistances as well as for quality or development traits. However, introducing wild genotypes into the classical breeding programs may have some advantages but also many drawbacks. Especially, these species carry numerous traits that are not compatible with modern agricultural practices such as plant height, poor lodging resistance, free threshing, etc. At the present time, new genomic tools that have been extensively developed for wheat genetics can be used in breeding programs for marker assisted selection, QTL detection or positional gene cloning. Nevertheless, these tools are not adapted to the study, exploration and exploitation of wild genetic resources, because of the low transferability of g-SSR markers. Because of the cost, almost no effort is made to develop genomic tools necessary for each species. The aims of the thesis were thus 1) to develop a set of markers useful for a large number of wild wheat-related species and 2) to use this set for phylogenetic studies.

3.5.2 Methodology

As demonstrated above, the SSRs are the marker of choice for wheat genetic studies but they are expensive to develop. According to this, it was concluded that developing a specific representative set for each species was prohibitive. In addition, the g-SSRs are only poorly transferable to related species and the markers developed from wild species will be useful neither on wheat nor on the other species. Concerning this latter point, sequences that are likely to be more transferable are the coding sequences because they are known to be well conserved even between divergent species. It was also demonstrated that a significant proportion (about 10%) of the genes contain a microsatellite motif. It was thus decided to develop a set of microsatellites issued from wheat coding sequences. At the present time, there are more than 600,000 wheat ESTs in the international data bases (http://www.ncbi.nlm.nih.gov/dbEST/) representing nearly 50,000 unique contigs suggesting that about 5,000 EST-SSRs can be developed. We also decided to use the available rice sequence (http://www.tigr.org/tdb/e2k1/osa1/pseudomolecules/info.shtml) to develop EST-SSRs from the rice coding regions. We decided to focus on rice chromosome

1 because the syntenic relationships are known to be well conserved with wheat group 3 chromosomes. This homoeologous group is of main interest for the UMR since the physical map of the chromosome 3B is currently under elaboration in the team. Moreover, this chromosome is the largest in physical size, and a number of important traits are known to be controlled by loci on this chromosome. Because we would like to have together transferable markers to a large range of related species and showing the highest possible variability, our EST-SSRs were tested on a set of more than 30 species and on about five accessions for each species.

3.5.3 Deliverables

According to the aims, the results will be presented into three parts:

-the development of the set of markers. From the experience of the lab, it was decided to start with a set of 300 ESTs containing a microsatellite motif. It was expected to have about 80% of the primer pairs giving an amplification product and about 40% giving polymorphism on wheat.

-the study of the transferability of the EST-SSRs to wheat-related species. This part was made following a two-steps procedure. Transferability was first evaluated on a set of eight different species. Then, the most transferable markers were used on the total set of the lines.

-the evaluation of genetic diversity within and between the species. Genetic diversity was estimated in terms on number of new alleles compared to wheat. Phylogenetic relationships between the species were then evaluated using either separated sets of markers (according to their chromosomal assignment) or the whole set but only between species with similar levels of ploidy.

MATERIALS & METHODS

Chapter II: Material and Methods

1 Plant material and DNA extraction

1.1 Hexaploid wheats for polymorphism evaluation

In a first phase, eight hexaploid wheat lines corresponding to the parents of five mapping populations were used for polymorphism screening of the microsatellites: W7984 (synthetic wheat, Van Deynze et al. 1995) and cultivars Opata, Courtot, Chinese Spring, Eurêka, Renan, Arche, and Récital. Twenty-two additional wheat cultivars (Annex 2) were further analysed in order to evaluated polymorphism information content (PIC) values and for phylogenetic studies. These lines were selected according to Roussel et al. (2005) in order to maximize the variability. They are issued from a larger core-collection of 372 lines which represents nearly the whole variability (98%) existing in the total collection available in Clermont-Ferrand (F Balfourier, personal communication). For each accession, ten seeds from self-pollinated ears were sown for further DNA extraction.

1.2 Grass species for transferability studies

Eight accessions of cultivated or wild species including polyploid as well as diploid species were primarily used to study the transferability of EST-SSRs to close and wild relatives of wheat: *T. durum*, *T. monococcum*, *Ae. speltoides*, *Ae. tauschii*, rye (*Secale cereale*), barley (*H. vulgare*), *Agropyron elongatum*, and rice (*Oryza sativa*). In a second step, a larger number of wheat-related species or sub-species was evaluated. In order to facilitate the comparison, similar numbers of accessions (between two and six) for each species were randomly chosen among our collection except for *T. durum* and Triticale where respectively 23 and 8 varieties were selected. Overall, 182 accessions representing 33 species or sub-species of the grass family and 17 genomes were used (Annex 2). Seeds were mainly obtained from the Centre of Biological Resources on Cereal Crops (INRA-Clermont-Ferrand), and from Jacques David (INRA Montpellier, tetraploid and *Aegilops* species), Nathalie Chantret (INRA-CIRAD Montpellier, rice species) and Philippe Barre (INRA Lusignan, *Lolium* species). Similarly as for wheat, for each species, between five and ten seeds from self-pollinated ears, (when available or possible) except for rye and *Ae. speltoides* were sown for further DNA extraction.

Table 2-1: List of the 19 nulli-tetrasomic (NT) and three ditelosomic (DT) lines used for chromosomal assignment of the EST-SSRs.

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
A genome	N1AT1B	2AS	N3AT3B	N4AT4B	N5AT5B	N6AT6B	N7AT7B
B genome	N1BT1A	N2BT2A	N3BT3A	4BS/4BL	N5BT5D	N6BT6D	N7BT7A
C genome	N1DT1A	N2DT2A	N3DT3A	N4DT4B	N5DT5A	N6DT6A	N7DT7A

1.3 Aneuploid lines

Hexaploid wheat tolerates the loss of a certain level of its genetic patrimony because this can generally be balanced by the other homoeologous chromosomes. Thus, stocks of aneuploid lines were developed from Chinese Spring by Sears (1954, 1966) and were kindly provided to us by Dr Steve Reader (John Innes Centre, United Kingdom) for chromosomal assignment of EST-SSR markers. The complete set of nulli-tetrasomic (19 NT, Table 2-1) lines except nulli 2A (N2A) and nulli 4B (N4B) supplemented with three ditelosomic (DT) lines (DT2AS, DT4BS, DT4BL) was used to assign marker to wheat chromosomes or chromosome arms.

1.4 Segregating populations

Two segregating populations extensively used in the laboratory and for which well-saturated genetic maps were previously elaborated, were used for genetic mapping of the polymorphic EST-SSRs.

1.4.1 ITMI population

The ITMI (International Triticeae Mapping Initiative) population is recognized as the international reference for genetic mapping. It was developed from the cross between the Mexican spring wheat cultivar Opata 85 and the Synthetic wheat W7984. This latter was obtained from a cross between the diploid D genome ancestor of cultivated wheat, *Aegilops tauschii* (Coss.) Schmal. and the CIMMYT (Centro International de Mejoramento de Maize Y Trigo) *T. durum* (AABB) variety ‘Altar 84’, using the *T. durum* line as the female parent. Crossing between these two species was followed by embryo excision and culture and colchicine treatment of seedlings in order to generate fertile amphihexaploid plants. Pollen from one of these was used to pollinate the variety ‘Opata 85’. The F1 progeny was grown in Ciudad Obregon, Mexico, and the F2 seeds were cultivated and advanced by single-seed descent to the F7-8 generation in Cornell University (New York, USA). A set of 114 lines was randomly selected and widely distributed in various labs all over the world for genetic mapping leading now to more than 3,000 loci mapped. In our case, we selected 90 lines for genotyping in order to integrate our EST-SSRs to ITMI reference map.

1.4.2 Courtot x Chinese Spring population

The population consisted in 217 doubled-haploid (DH) lines and was produced through

anther culture from Courtot (Ct) x Chinese Spring (CS) F_1-hybrids (Félix et al., 1996; Cadalen et al., 1997) according to the procedure described in Martinez et al. (1994). Contrary to Chinese Spring, Courtot is a semi-dwarf variety with a good productivity and a good bread-making quality. However the former represents the international reference in wheat genetics with numerous series of specific material (Sears 1954, 1966; Law et al. 1988; Gale and Miller 1988; Endo and Gill 1996) and a lot of Expressed Sequence Tags (ESTs) and full-length cDNA libraries (Ogihara et al. 2004). Moreover, these two cultivars presented a high level of polymorphism (60%, Cadalen et al. 1997) as well as many differences for several agronomic traits (for a review see Sourdille et al. 2003). One hundred and six lines were genotyped for most markers and an additional set of 81 DH lines were genotyped only for anchor loci. The genetic map now includes 824 loci and covers 3,685 cM (Sourdille et al. unpublished results). Ninety-four DH lines were screened in our study to integrate our EST-SSRs into the CT x CS genetic map.

1.5 DNA extractions

DNA extraction was performed according to a CTAB protocol as described by Murigneux et al. (1993). The detailed protocol is presented in Annex 6. About 3g of fresh young leaves of each individual were cut from 3- to 4-weeks-old seedlings and were ground in liquid nitrogen. The quality of each DNA sample was estimated after migration on 0.8% agarose gel and their concentrations were quantified using spectrophotometry at $OD_{260\ nm}$ (Eppendorf, BioPhotometer, Germany).

2 Wheat and rice EST-SSRs

2.1 Analysis of the wheat ESTs

The clustering was done from 170,746 wheat EST sequences originating from public and Génoplante databases (92,387 and 78,359 ESTs, respectively). The sequences were previously masked for vectors and repeats. The minimal overlapping length was fixed at 80 bp with 96% identity for an alignment length between 80 bp and 200 bp and 90% identity for an alignment length greater than 200 bp. The CAP3 program (Huang et al. 1999) was used for EST assembly with default parameters on unmasked sequences. This resulted in 19,191 contigs containing a range of 2–395 sequences and 27,319 singletons (Nicot et al. 2004). The 46,510 consensus sequences ranged from 93 to 7,497 bp in length and were screened for the presence of SSRs using either REPEATMASKER (Smit AFA and Green P,

http://ftp.genome.washington.edu/RM/RepeatMasker.html) SSRSEARCH (ftp://ftp.gramene.org/pub/gramene/software/scripts/ssr.pl) or TRF (Benson 1999) programs. A microsatellite was considered when the sequence contained a minimum of three repeats of a motif comprising from one to six nucleotides, with a total length of at least 12 nucleotides. ESTs containing SSRs were then extracted from the pool of 46,510 contigs. For our study we randomly selected about 1,000 EST sequences containing a microsatellite which were further used for primer design.

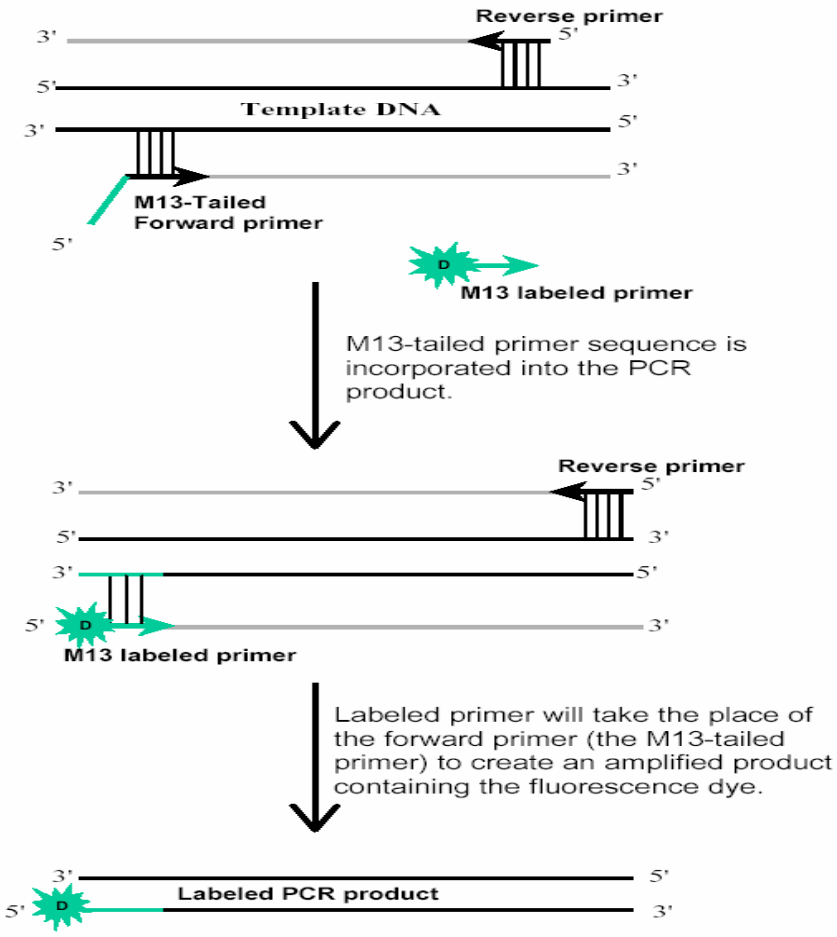
2.2 Primer design

Primers were designed using Primer software (version 0.5, Whitehead Institute for Biomedical Research, Cambridge, Mass.) based on the following criteria: primer length ranging from 18-22 bp with 20 bp as the optimum; product size ranging from 100-400 bp; melting temperature (Tm) between 57-63°C with 60°C as optimum; GC% content between 20-80%; maximum acceptable primer self-complementarity of 5 bases; maximum acceptable 3' end primer self-complementarity of 3 bases. Primers were selected when they contained as few as possible of repeated sequence, and when the 3'-end of the two primers ended with C or G when possible. Primer sequences were subjected to BLAST analysis against an in-house database to avoid redundancy with those that already exist. On this basis, 301 primer pairs were selected, and designated as CFE (primer sequences available on Graingenes: http://wheat.pw.usda.gov/index.shtml). Each forward primer was M13-tailed (M13: 5'CACGACGTTGTAAAACGAC3', synthesis MWG (Germany)) for easier automation on capillary electrophoresis system (see further).

2.3 Rice EST-SSRs

Synteny relationships between rice and grass species using a circle as a model were already described by Moore et al. (1995) and colinearity between wheat group 3 chromosomes and rice (*Oryza sativa* L.) chromosome 1 was even shown earlier (Devos et al. 1992; Ahn et al. 1993; Kurata et al. 1994; Van Deynze et al. 1995) and precised recently (Sorrells et al. 2003; La Rota and Sorrells 2004, Munkvold et al. 2004). We thus decided to develop SSRs from rice chromosome 1 and test for their transferability on wheat group 3 chromosomes. From TIGR rice pseudo-molecules, (The Institute for Genomic Research; http://www.tigr.org/, version V02) 6,852 unigene sequences from rice chromosome 1 (including exons, introns and upstream/downstream untranslated region) were screened for the presence of perfect and imperfect SSRs using either SSRSearch or TRF programs

Figure2-1. M13-tailed primer strategy



according to the same criteria as described above (3.1). More than 4,000 gene sequences located on rice chromosome 1 contained at least one SSR. Based on the ARTEMIS viewer, we selected 200 rice genes bearing SSRs, half of them being collinear to the wheat 3BL7-0.63-1.00 long arm distal deletion bin and the remaining 100 genes corresponding to the bin 3BL2-0.22-0.50. These two regions are known to show a better level of conservation with wheat homoeologous group 3 (Munkvold et al. 2004). The sequences were then analyzed using Vector NTI (version 7) software to identify the location of the SSR in CDS or in intron and only those where SSRs located in CDS were retained in order to maximize the probability of conservation and thus transferability between rice and wheat. Primers were designed using the following criteria: Tm ranging from 40 to 50^{o}C, GC% from 35 – 60 %; primer length from 20 – 25bp; maximum nucleotide repeats of 4; maximum palindromes of 8; stem length of hairpin loop ≥ 3 and permitted dG ≥ -1.0; the different Tm between forward and reverse primer ≤ 5^{o}C; the different GC% content ≤ 10; permitted dimers with dG ≥ -15 kcal / mol and permitted primer-primer 3'end complementarities with dG ≥ -3 15 kcal / mol. Primers were selected when forward and reverse primer showed as similar as possible Tm and GC% and formed as few as possible dimers, and when the 3'-end of the two primers ended with C or G whenever possible. On this basis, 106 primer pairs were selected, and designated as CFR. Like for wheat EST-SSRs, each forward primer was M13-tailed.

2.4 EST-SSR detection

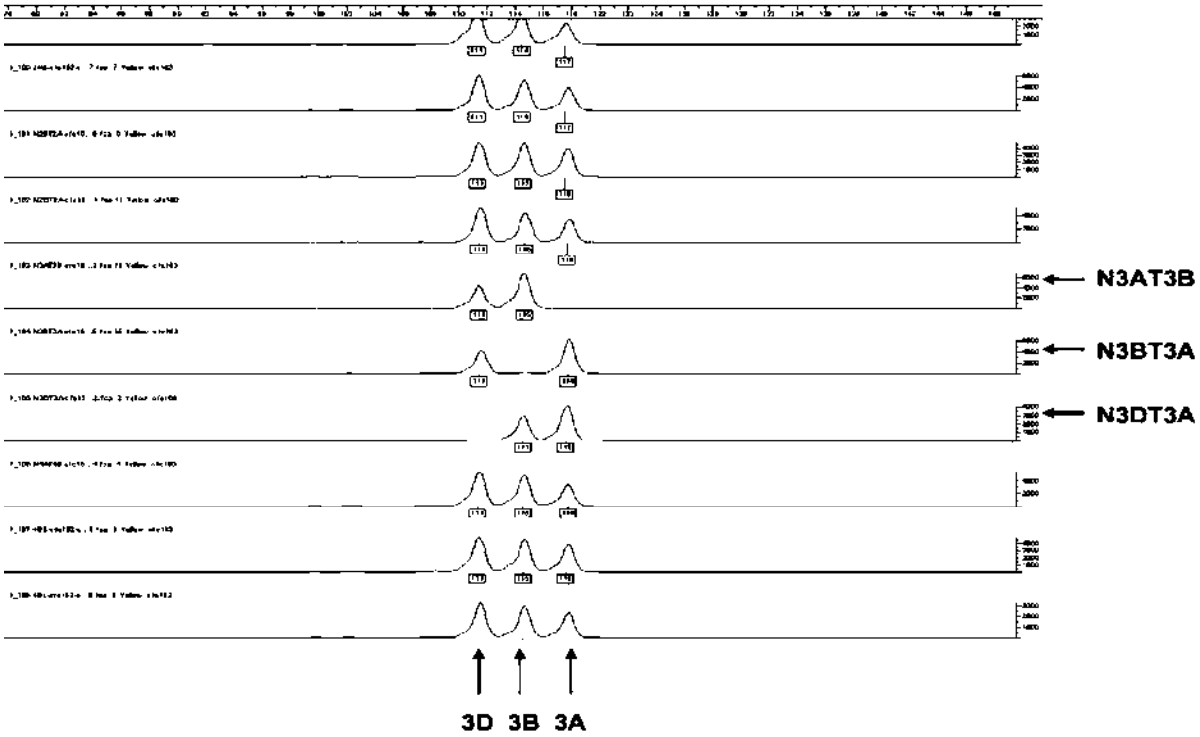
2.4.1 Principle of the M13-tailed primer method

For automation of the SSR detection on the ABI3100 capillary electrophoresis system (Applied Biosystems), DNA fragments must be labeled with specific fluorescent dyes. Because labeling all the primers individually would prohibitively increase the cost of genotyping projects, which involve many SSR markers, an alternative and cost-effective method was used. This technique employed a two-part primer in which a standard "tail" corresponding to a universal M13 primer is added to the 5' end of the forward primer sequence. The amplification of SSRs is thus performed with three primers: the forward primer with the M13 tail, the reverse non-tailed primer and the fluorescently labeled M13 primer (Boutin-Ganache et al 2001; Zhang et al. 2003; Fig 2-1).

2.4.2 PCR reaction and detection

PCR reactions were performed in two types of plate (96- or 384-wells plates). For the

Figure2-2: Example of chromosomal assignment for Xcfe 163 by aneuploid analysis



96-wells-plate, a volume of 20 µL containing 80 ng of template DNA, 200 µM of each dNTP, 0.4 unit of Taq polymerase (Qiagen) with 1X of its appropriate buffer (1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0) and 50 mM KCl), 500 nM of each M13-labeled (6-FAM, HEX, VIC, PET or NED) and reverse primer, 50 nM of the forward M13-tailed primer, was added into each well. For the 384-well-plate, PCR reactions were carried out in a final volume of 6.5 µL with the same concentrations as above except that only 25 ng of template DNA and 0.2 U of Taq polymerase (Qiagen) were used. PCR amplifications were conducted using the following procedure: 5 min 95°C, followeb by 20 cycles (30 sec 94°C, 30 sec 60°C, 30 sec 72°C) and 5 min 72°C. Fluorescent amplification products were then mixed with de-ionized formamide and an internal size standard (Genescan 400 or 500–TAMRA, Applied Biosystems) in a 1:12:0.5 volume ratio. After a 5 min denaturation at 95°C, amplification products were visualized using an ABI PRISM®3100 Genetic Analyzer (Applied Biosystems). Fragment sizes were calculated using GENESCAN and GENOTYPER softwares (Applied Biosystems), where different alleles are represented by different amplification sizes for tandem repeats.

3 Data analysis

3.1 Genetic and cytogenetic mapping of EST-SSRs

3.1.1 Chromosomal assignment

Those primer pairs that yielded products were used for amplification on the 19 nulli-tetrasomic (NT) and three ditelosomic (DT) lines derived from Chinese Spring (Fig 2-2). EST-SSRs loci were assigned to the chromosomes corresponding to the nullisomic-tetrasomic lines for which no amplification product was obtained, following confirmation that all other nullisomic-tetrasomic lines amplification products were in order. An example of chromosomal assignment for Xcfe163 is shown in Figure 2-2.

3.1.2 Genetic mapping

The polymorphic EST-SSRs were genotyped on either the ITMI or the CtCS segregating populations according to the polymorphism revealed. The data were integrated into an existing framework map. For all loci, goodness of fit to a 1:1 segregation ratio was tested using a chi-square analysis. Linkage maps were previously constructed with MAPMAKER/exp 3.06 (Lander et al. 1987) for loci showing no segregation distortion (Sourdille et al. 2003 and unpublished results). Linkage groups were established by

calculating recombination frequencies with identical conditions (maximum recombination fraction = 0.35 and minimum LOD score = 3.0). The Kosambi mapping function (Kosambi 1944) was applied to transform recombination frequencies into additive distances in centiMorgans. In our case, the new EST-SSR loci were attributed to and placed within the framework of the chromosomes using respectively the “assign” and “place” commands of Mapmaker.

3.2 Transferability of the EST-SSRs to wheat related species

Transferability of the wheat EST-SSRs to the related species was computed as the percentage of markers giving an amplification product on at least one of the accessions from a given species or sub-species. As an estimate of the genetic diversity within each species or sub-species, the number of bands per locus and per individuals (NB) was computed as follows: NB = (N x 100)/(116 x Transferability (%) x Number of individuals) where N is the total number of bands observed for each species, 116 is the total number of EST-SSRs tested and Transferability is the percentage of EST-SSRs that give an amplification product on related species.

3.3 *In silico* analysis of EST-SSRs

To assign putative functions to EST bearing SSRs, the sequences were compared to the SwissProt and TrEMBL protein databases using BLASTx algorithms (Altschul et al. 1990), with expected value of 1 e^{-5} as a significant homology threshold. Putative functions were attributed according to the definitions given at http://www.godatabase.org/cgi-bin/amigo/go.cgi. Three different classes of function are proposed: (1) biological process, which means phenomena, marked by changes that lead to a particular result, mediated by one or more gene products, such as light-inducible protein CPRF-2. (2) Cellular components including gene products that are parts of macromolecular complexes, such as Actin. (3) Molecular function which means elemental activities, such as catalysis or binding, presenting the actions of a gene product at the molecular level. For example, sulfite reductase (Ferredoxin) involves in oxidoreductase reaction in catalytic activity. tBLASTx searches were also performed against rice and barley NCBI unigene sets (http://www.ncbi.nlm.nih.gov/) to study the degree of conservation of the repeated motif between the three species. In addition, the EST sequences were compared to the rice pseudo-molecules (http://www.tigr.org/tdb/e2k1/osa1/, version V01) using tBLASTx and BLASTn to identify putative orthologues on rice chromosomes. E-values of less than 1 e^{-5},

1 e^{-10}, 1 e^{-25}, 1 e^{-50} and 1 e^{-100}, were selected.

3.4 Distribution of EST-SSRs on the rice genome

Potential bias in the distribution of wheat EST orthologs on rice pseudomolecules was investigated using the relative error (Er). This value was computed as follows: the rice gene proportion for each chromosome was calculated as the ratio between the number of genes on the rice chromosome and the total number of genes on the rice genome (values at http://www.tigr.org/tdb/e2k1/osa1/). Then the expected number of hits was evaluated as the product of the number of wheat EST blasted (251) by the rice gene proportion. Observed hit values were compared to the expected values using a classical binomial test from Splus and only significant values at the 0.05 or 0.01 thresholds were retained.

3.5 Determination of Polymorphism Information Content (PIC) values

For each EST-SSR, the numbers and the frequencies of alleles were computed. Two alleles are considered as identical when they show the same fragment size. The polymorphism information content (PIC) value (Nei 1973) was then computed for each marker using the following formula:

$$PIC= 1-\sum (Pi)^2$$

where P*i* is the proportion of the population carrying the i^{th} allele, calculated for each SSR locus. This value provides an estimate of the discriminating power of a locus by taking into account not only the number of alleles that are expressed but also their relative frequencies. PIC values were only computed for *T. aestivum*, *T. durum* and Triticale species because larger numbers of accessions were used.

3.6 Determination of the Jaccard similarity coefficient (1908)

For phylogenetic studies, the binary matrix was generated as followed: presence of an amplified product of a given size was scored as "1" while the absence of the same amplification product was scored as "0". The binary data were used to compute the distance matrix as 1 – the Jaccard's similiraty coefficient (1908). This latter coefficient (s_{ij}) measures the asymmetric information on binary variables and is computed according to the following formula:

$$s_{ij} = \frac{p}{p+q+r}$$

Where p = number of bands present in both individuals (i and j)

q = number of bands present in i and absent in j

r = number of bands present j and absent in i

Because transferability was not complete, null alleles were not considered and were quoted as missing data since there was a higher probability that the lack of amplification was due to the presence of numerous mutations in the flanking sequences which are obviously different between the species rather than to a deletion of the genes which could have been considered as similar events. This also justifies the choice of the Jaccard distance index, which does not consider as informative a shared absence of a given trait (here an amplification product).

3.7 Unweighted Pair Group Method with Arithmetic Mean (UPGMA)

There are numerous methods for constructing phylogenetic trees from molecular data (Felsenstein 1988; Miyamoto and Cracraft 1991). In the present study, a phylogenetic tree was constructed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA, Phylip software (Felsenstein et al. 1993)), which is the simplest, and the most intuitive method for tree construction. As all the species studied belong to the *Triticeae* tribe, the assumption of a molecular clock was acceptable. Therefore, the trees obtained by the UPGMA clustering method can be considered as phylogenetic inferences. The reliability and goodness of fit of dendrograms obtained from EST-SSRs data were tested through bootstrapping based on 100 samples (Felsenstein, 1985). This led to 100 phylogenetic trees summarized in a consensus tree which indicated the proportion of bootstrapped trees showing that same clade.

RESULTS & DISCUSSION

Figure 3-1: Distribution of the different types of SSRs in wheat EST-SSRs.

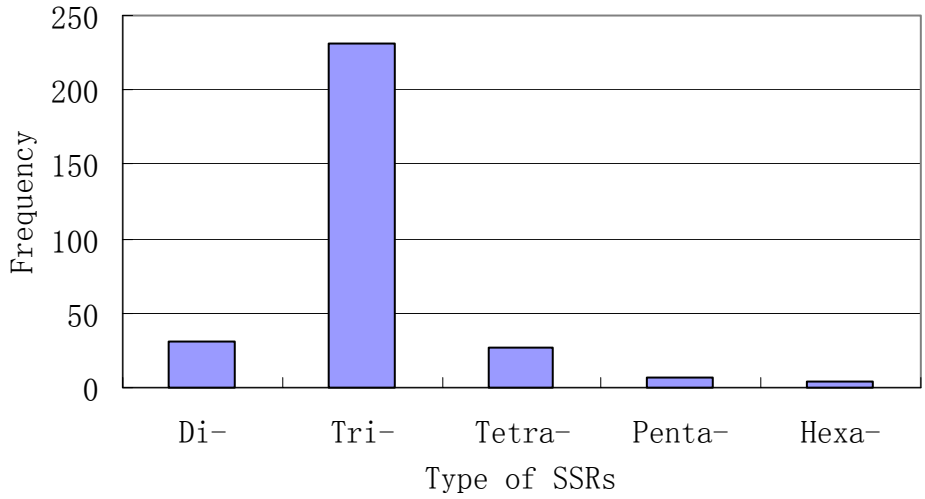
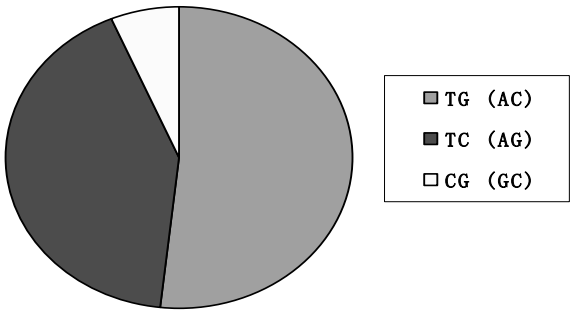
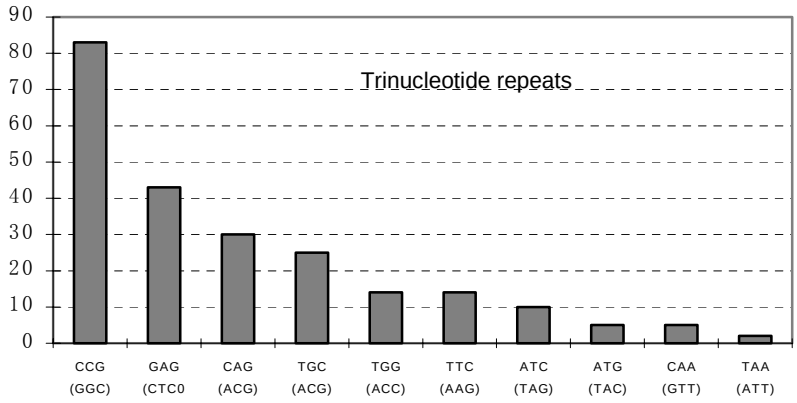


Figure 3-2: Frequency of the motifs according to their respective length. a: dinucleotide motifs; b: trinucleotide motifs; c tetranucleotides motifs; d: pentanucleotide motifs; e: hexanucleotide motifs.

a:



b:



Chapter III: Results and discussion

1 Analysis of the wheat ESTs

All of the CFE forward and reverse primers designed within this study are listed in Annex 3.

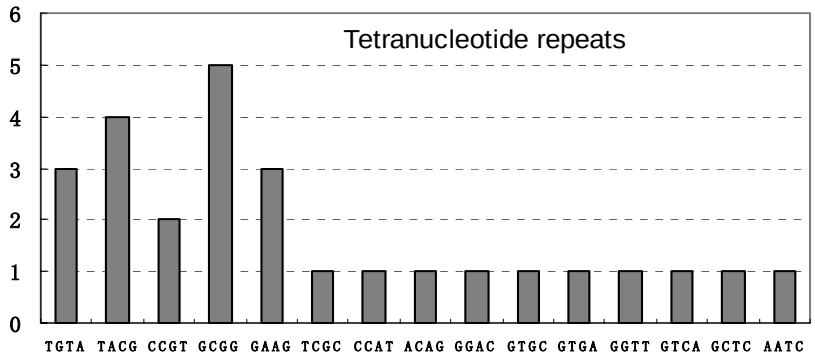
1.1 Characteristics of EST-derived SSRs

Among the 301 wheat EST-derived SSRs selected, five different classes of repeated motif length occurred (di- to hexanucleotides). The trinucleotide repeats were the most common in our wheat ESTs (77%), followed by dinucleotide and tetranuleotide motifs accounting respectively for 10.33% and 9%. The hexanuleotide motifs were the least common, accounting for only 1.3% (Figure 3-1). Almost all EST-SSRs (99.3%) except two (Xcfe58 (TTA), Xcfe197 (TAA)) contained either a G or a C in the motif, whereas only 48% of genomic SSRs did so (Wang et al. 1994).

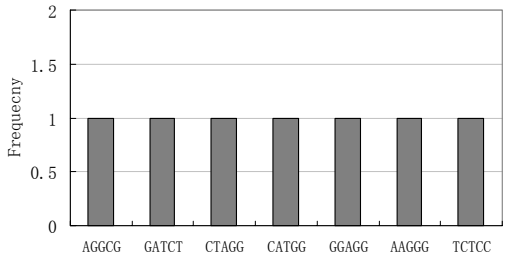
Concerning dinucleotide repeats, only three occurred: (TG)n, (TC)n and (GC)n with frequencies of 51.6%, 41.9% and 6.5% respectively (Fig 3-2a). No (TA)n motif was observed in our case. For trinucleotide repeats, (GCC)n motif was the most abundant, representing 36% followed by the (GAG), (CAG), (TGC), (TGG), (TTC), (ATC), (ATG), (GAA) and (TAA) (Fig 3-2b). Frequency for tetranucleotide repeats are illustrated in Figure 3-2c. Fifteen among the 33 possible classes of tetranucleotide repeats occurred in this study, (GCGG) being the most frequent followed by (TACC). Only seven different pentanucleotide repeats were found (AGGCG, GATCT, CTAGG, CATGG, GGAGG, AAGGG, TCTCC) and only three hexanucleotide repeats (GAACCC, GGCGGT, GGGGGC). Results are shown in Figure 3-2d and 3-2e.

The frequency of the number of repeats (n) was also surveyed (Figure 3-3). SSRs with large numbers of repeats were less frequent than those with low numbers of repeats. Motifs with four repeats occurred the most frequently in wheat EST-SSRs, accounting for 37.4% of the n values. Most of the markers showed repeat unit numbers lower than 10 and contributed 94.5% to the total number of EST-SSRs.

c:



d:



e:

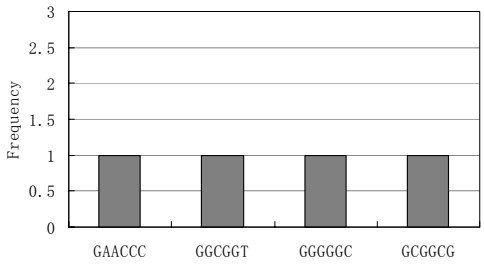
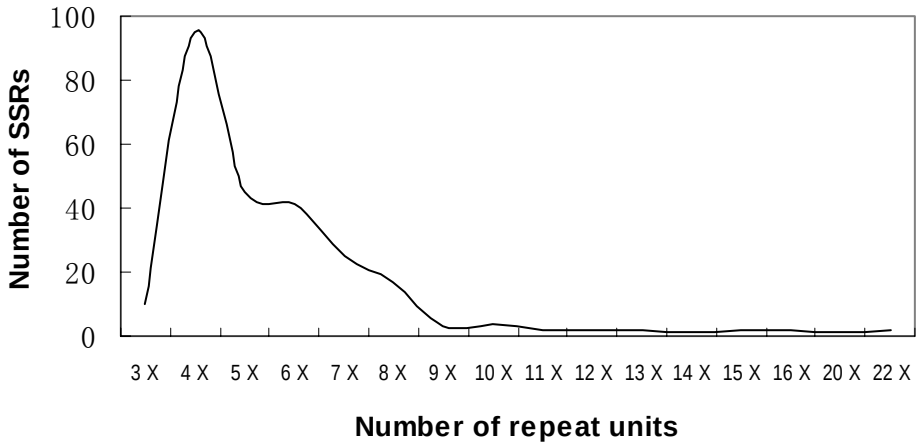


Figure 3-3: Distribution of the SSRs according to the number of repeats



1.2 Amplification and polymorphism of EST-SSRs

The 301 primer pairs were tested on eight reference cultivars in order to detect polymorphism. For each primer pair, quality of the amplification, real size of the PCR product, and polymorphism between the parents of four mapping populations are shown in Annex 4. About 80% (240) of the primer pairs led to an amplification product. Some differences were observed between the expected and the observed size of the amplification product. Among the 240 primer pairs, 51 (21.3%) gave an amplification product either larger, suggesting the possible simultaneous amplification of an intron during the PCR, or smaller than expected, suggesting (1) the occurrence of deletions within the genomic sequences, (2) a lack of specificity of some primer pairs which may have amplified a different copy belonging to the same multigenic family, or (3) a slight variation between the amplified copy and the consensus sequence.

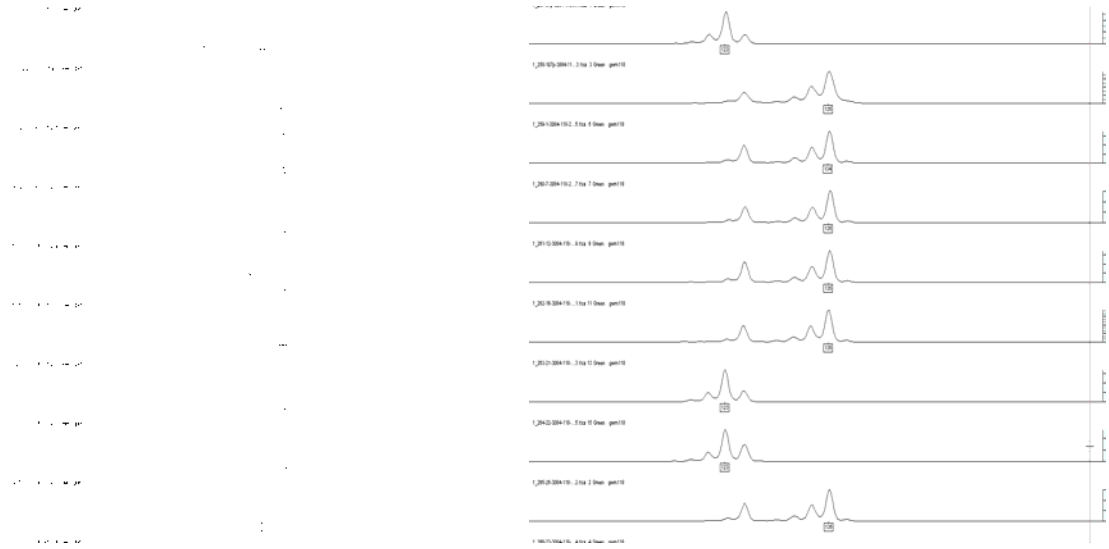
In general, EST-SSRs markers produced high quality patterns compared to those from genomic SSRs (Fig. 3-4). In our study, 192 markers produced strong and clear bands, representing about 80% of markers giving amplification (240).

Among all the cultivars tested, for all of EST-SSR markers, dinucleotide repeats displayed higher level of polymorphism (Tab 3-1, 90.5%) than trinucleotide repeats (80%), while tetranucleotide repeats showed the lowest level (57%). At the same time, 139 (58%) of the 240 markers giving an amplification product showed polymorphism on at least one of the eight wheat cultivars used. The combination W7984 x Opata (37.5%) was the most polymorphic (Figure 3-5) followed by Courtot x Chinese Spring with 25.4%, whereas Eurêka x Renan and Arche x Récital gave the lowest level of polymorphism with 20.8% and 20% respectively. The average level of polymorphism was 25.4% for the four populations.

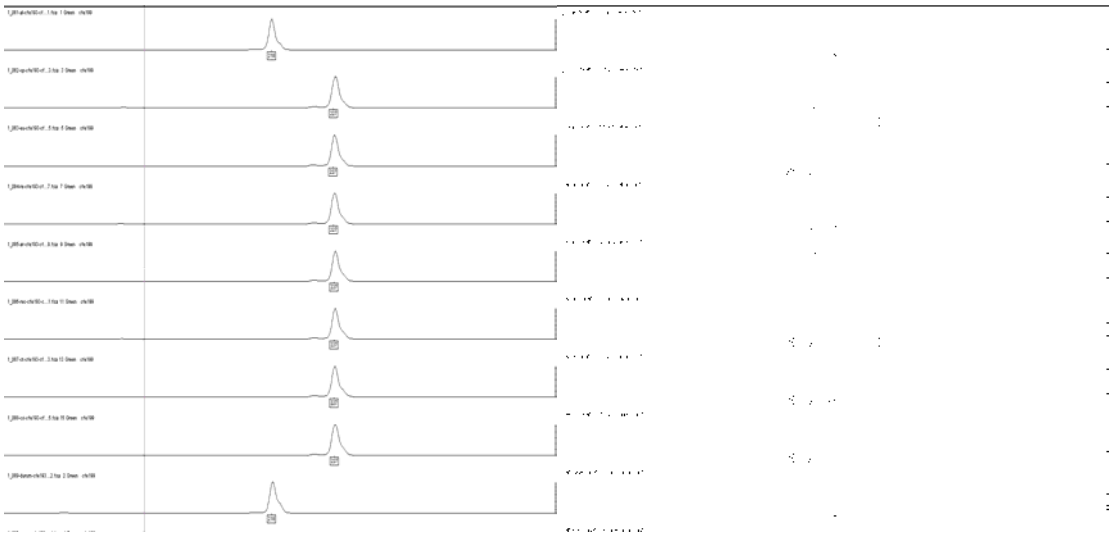
Overall, 223 out of the 240 primer pairs that amplified distinguishable bands in wheat cultivars gave clear and readable patterns and were used to survey the number of bands produced. Most of them (96%, Figure 3-6) gave less than four amplification products. About 19.7% gave three bands probably corresponding to the three homoeologous chromosomes while 54 % gave only one band suggesting that these latter were specific of one of the three copies or that the three copies had identical sizes or that these SSRs were located within unique genes.

Figure 3-4: Comparison patterns produced by g-SSRs with those by EST-SSRs

a. Patterns produced by g-SSRs Xgwm383 (L) and Xgwm118 (R)



b: Patterns produced by EST-SSRs Xcfe190 (L) and Xcfe257 (R)



1.3 Genetic mapping of the EST-SSRs

According to the survey of polymorphism, 90 out of the 240 EST-SSRs showed polymorphism between the two parental lines of the ITMI population, Synthetic W7984 and Opata 85. Only 71 markers gave useful genotyping in the segregating population because: i) some makers gave too weak bands to accurately distinguish the parental alleles; ii) some that exhibited polymorphism between the parents were no more polymorphic within the population. Among these 71 markers, 65 gave clear segregations and generated 75 loci which were tentatively integrated into the ITMI map. The remaining six were excluded because of too complex patterns. Finally, 62 loci were directly integrated to the map (Annex 5) while the remaining 13 loci were found to be unlinked to any of the other markers. Eighteen additional markers that did not show any polymorphism in the ITMI population were polymorphic between Courtot and Chinese Spring and could therefore be mapped on the CtCS population. These markers gave rise to 22 loci among which, 19 were added to the CTCS map (Annex 5), three remaining unlinked to any linkage group. Results of the assignment to the different chromosomes are summarized in Tab 3-2. While no significant differences were observed concerning the chromosomal assignment of the wheat EST-SSRs to the wheat chromosomes (see further details in Zhang et al. 2005 below), more loci mapped to the B genome (40) compared to the A (31) and especially to the D (10) genomes confirming that the former exhibits more polymorphism than the other two. Similarly, only three loci mapped to group 5 chromosomes suggesting a better conservation and less polymorphism within the genes from these chromosomes compare to the others. For the map position (Annex 5), most of the loci were located in the distal regions of wheat chromosomes, and only 13 loci were localized close to the centromeres. However, the proximal genetic location does not involve that these markers are physically located close to the centromere since genetic to physical distance relationships may vary a lot along the chromosome (Sourdille et al. 2004).

1.4 Discussion

Recent studies on several plant genomes have demonstrated that SSRs were predominantly located in ESTs compare to genomic DNA (Morgante et al. 2002). We thus investigated the frequency of different classes and types of SSRs in wheat ESTs. Tricluneotide repeats were the most frequent (~77%). This was expected since these SSRs only change the number of amino acids in the protein but do not modify the reading frame if one or more motifs are

Table 3-1. Level of polymorphism displayed by the different types of SSR repeats.

Repeated motif	N. of amplification	N. of polymorphism	Percentage
Dinucleotides	21	19	90.5%
Trinucleotides	129	103	80%
Tetranucleotides	21	12	57%

Figure 3-5: Level of polymorphism observed for each of the four segregating populations available for mapping in the laboratory. W-Op: W7984 x Opata (ITMI population); Eu-Re: Eurêka x Renan; Ar-Réc: Arche x Récital; Ct-Cs: Courtot x Chinese Spring; Average: average level of polymorphism for the four mapping populations.

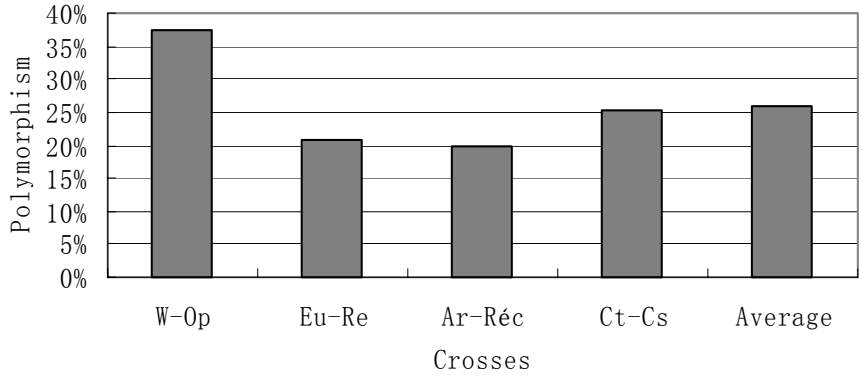
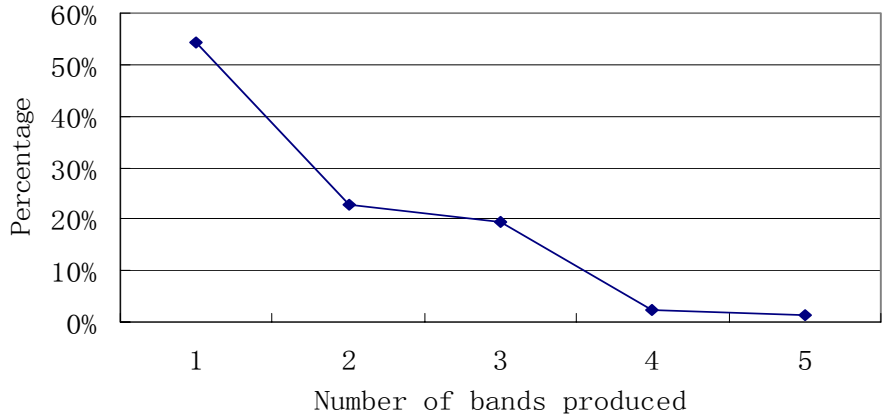


Fig 3-6 Distribution of number of bands amplified by wheat EST-SSRs in 8 wheat cultivars



added or deleted. This was also consistent with the results reported earlier (Morgante et al. 2002; Varshney et al. 2002; Nicot et al. 2004). Within EST-SSRs, the abundance of trinucleotide repeats is attributed to the suppression of specific selection against the frame shift mutation in coding region resulting from length changes in nontriplet repeats (Metzgar et al. 2000)

For trinucleotide motifs, (CCG)n was very abundant in wheat EST sequences although Kantety et al. (2002) found that in wheat, the most abundant trinucleotide motifs were AAC/TTG. However, this was consistent with the results of Morgante et al. (2002) and of Murray et al. (1989) who found a high GC% level in the monocotyledonous genes. Among the ESTs analysed, 21% were issued from seed tissues. In seeds, the most frequent amino-acids are Proline (Pro) and Glutamine (Gln) (Feillet, 1965). Pro is coded by CCG and Gln by CAA and CAG. These three motifs represent 60% of all the trinucleotide microsatellites we found in wheat ESTs. The difference observed with Kantety et al. (2002) was probably due to the tissues used for EST production. The two motifs ATT/TAA and CTA/TAG were less represented (0.54% and 1.36% respectively) presumably because they constitute stop codons.

We found only two EST-SSRs without any C or G. Wang et al. (1994) demonstrated that the majority of SSRs in coding region contained G+C base pairs, contrarily to A+T base pairs, and also found that all of 8 (ATT)n, (TTA)n repeats were in non-coding region. Our results were in agreement with those of Wang et al. (1994). The presence of “CpG islands” upstream of many genes can be used to explain this phenomenon. Besides, due to the stronger linkage between G and C than between A and T, this ensures a correct replication (Carels et al. 2000). In addition, that TAA is a stop codon can also partially account for this phenomenon.

Most wheat EST-SSRs were shorter than 10 repeat units and four units were the most frequently encountered in our research. Lot of studies using both *in silico* analysis and experimental designs showed that this feature is common to many species (Kantety et al. 2002; Thiel et al. 2003; Barrett et al. 2004; Varshney et al. 2002). Maybe this explains that the polymorphism is lower for EST-SSRs than for genomic SSRs since longer SSRs are more polymorphic (Thuillet et al. 2004). Conversely the selection pressure is probably very strong and prevents the EST-SSRs from being too long and too polymorphic in order to keep them functional.

About 25% of the EST-SSRs exhibited polymorphism which was similar to the results

Table 3-2: Distribution of EST-SSR loci on the genetic maps (in brackets number of loci on ITMI and CtCS maps respectively) according to their assignment to wheat chromosomes and homoeologous groups

Homoeologous Group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	Total
Chromosome **A**	5 (0/5)	1 (1/0)	1 (1/0)	10 (10/0)	2 (1/1)	6 (4/2)	6 (4/2)	31 (21/10)
Chromosome **B**	9 (7/2)	5 (4/1)	9 (8/1)	2 (2/0)	0	4 (0/4)	11 (11/0)	40 (32/8)
Chromosome **D**	3 (3/0)	1 (1/0)	2 (2/0)	1 (1/0)	1 (0/1)	2 (2/0)	0	10 (9/1)
Total	17 (10/7)	7 (6/1)	12 (11/1)	13 (13/0)	3 (1/2)	12 (6/6)	17 (15/2)	81 (62/19)

described earlier (Eujayl et al. 2001, Gupta et al. 2003, Thiel et al. 2003, Gao et al. 2004, Nicot et al. 2004), and was much lower than that observed for genomic SSRs (53%, Eujayl et al. 2001). The characteristics of EST-SSRs, *i. e.* the existence in the coding region where the mutation was restrained and a lower number of repeat units, probably accounted for this lower percentage of polymorphism. We also found that dinucleotide repeat displayed a higher level of polymorphism than that of trinucleotide repeat, which was consistent with the results of Nicot et al. (2004).

Eighty one new EST-SSR loci were integrated to the two reference genetic maps currently used in the lab (62 and 19 on ITMI and 1 CtCS maps respectively). More loci mapped to the B genome which was expected since this genome is always reported as being more polymorphic than the other two. We noticed few discrepancies between cytogenetical assignment to chromosomes or chromosome arms using NT/DT lines and genetic mapping which could be attributed to the difficulty in clearly assigning the polymorphic locus or in a wrong placement on the genetic map. The EST-SSR loci were mainly distributed on the distal ends of the chromosomes which was consistent with what is observed in our *in silico* maps. Similar results were also reported in tomato (Areshchenkova and Ganal 2002) in rye (Khlestkina et al 2004) and in wheat (Gao et al. 2004).

As a conclusion, wheat EST-SSRs markers exhibited some interesting features, different from those of genomic SSRs. This makes them highly valuable as a source for marker development as well as other applications.

2 Transferability of the wheat EST-SSRs to grass species

This part was the subject of two manuscripts which were respectively published in Theoretical and Applied Genetics (111: 677-687) and accepted for publication in Plant Breeding. These papers deal with two of the scientific questions which were asked at the beginning of the project and which concern the transferability of wheat EST-SSRs to related species and the potential of these markers to serve as genomic tools for orphan species and to reveal the diversity existing within these species.

2.1 High transferability of bread wheat EST-derived SSRs to other cereals (2005, Theor Appl Genet 111: 677-687)

Theor Appl Genet (2005) 111: 677–687
DOI 10.1007/s00122-005-2041-5

ORIGINAL PAPER

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High transferability of bread wheat EST-derived SSRs to other cereals

Received: 23 February 2005 / Accepted: 14 April 2005 / Published online: 21 July 2005

Abstract The increasing availability of expressed sequence tags (ESTs) in wheat (*Triticum aestivum*) and related cereals provides a valuable resource of non-anonymous DNA molecular markers. In this study, 300 primer pairs were designed from 265 wheat ESTs that contain microsatellites in order to develop new markers for wheat. Their level of transferability in eight related species [*Triticum durum*, *T. monococcum*, *Aegilops speltoides*, *Ae. tauschii*, rye (*Secale cereale*), barley (*Hordeum vulgare*), *Agropyron elongatum* and rice (*Oryza sativa*)] was assessed. In total, 240 primer pairs (80%) gave an amplification product on wheat, and 177 were assigned to wheat chromosomes using aneuploid lines. Transferability to closely related Triticeae species ranged from 76.7% for *Ae. tauschii* to 90.4% for *T. durum* and was lower for more distant relatives such as barley (50.4%) or rice (28.3%). No clear putative function could be assigned to the genes from which the simple sequence repeats (SSRs) were developed, even though most of them were located inside ORFs. BLAST analysis of the EST sequences against the 12 rice pseudo-molecules showed that the EST-SSRs are mainly located in the telomeric regions and that the wheat ESTs have the highest similarity to genes on rice chromosomes 2, 3 and 5. Interestingly, most of the SSRs giving an amplification product on barley or rice had a repeated motif similar to the one found in wheat, suggesting a common ancestral origin. Our results indicate that wheat EST-SSRs show a high level of transferability across distantly related species, thereby providing additional markers for comparative mapping and for following gene introgressions from wild species and carrying out evolutionary studies.

Communicated by J.W. Snape

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Introduction

Simple sequence repeats (SSRs), also referred to as microsatellites, are tandem arrays of short DNA repeats that range rom 1 bp to 6 bp in length. Polymorphism, which is based on the differences in the number of DNA repeats at any given locus, is easily detected by PCR and can be classified into two classes based on origin: genomic SSR markers, which are developed from enriched genomic DNA libraries, and expressed sequence tag (EST)-SSRs, which are derived from EST sequences originating from the expressed region of the genome.

Genomic SSR markers were developed during the early 1990s. They are distributed throughout the genome and have been used extensively for genome mapping, DNA fingerprinting and a wide range of genetic diversity, population and evolutionary studies in both plant and animal species (Liu et al. 1996; Senior et al. 1996; McCouch et al. 1997; Röder et al. 1998; Gupta and Varshney 2000; Prasad et al. 2000; Ramsay et al. 2000). The numerous advantages of this type of marker, including their abundance and dispersion throughout the entire genome, high information content, co-dominant inheritance, reproducibility and genomic specificity, are well-documented (Morgante and Olivieri 1993; Rafalski and Tingey 1993; Powell et al. 1996). However, most genomic SSRs have neither a genic function nor close linkage to coding regions (Metzgar et al. 2000), they are very time- and cost-expensive to develop and they show only a limited transferability to related species (Sourdille et al. 2001; Guyomarc'h et al. 2002a, b).

EST-SSRs have received much attention recently because of the increasing amounts of ESTs being deposited in databases for various economically important plants, such as rice (*Oryza sativa*), maize (*Zea mays*), wheat (*Triticum aestivum*), sorghum (*Sorghum*

bicolor), barley (*Hordeum vulgare*) and rye (*Secale cereale*). EST-SSRs can be rapidly developed from the in silico analysis of EST databases at low cost, and due to their presence in expressed regions, they can lead to the development of gene-based maps which may increase the efficiency of marker-assisted selection (MAS) through the use of candidate genes. Assessments of the polymorphism, diversity and transferability of EST-SSRs have been carried out in rice (Cho et al. 2000), grape (Scott et al. 2000), sugarcane (Cordeiro et al. 2001; Grivet et al. 2003), tomato (Areshchenkova and Ganal 2002), loblolly pine (Liewlaksaneeyanawin et al. 2004), Alpine Lady-fern (Woodhead et al. 2003), pasture grass endophytes (Jong et al. 2002), barley (Thiel et al. 2003) and rye (Hackauf and Wehling 2002). In wheat, in silico analysis showed that the frequency of EST-SSRs is 1 at every 6.2 kb of EST sequence (Varshney et al. 2002). With the rapid increase in bread wheat ESTs in the databases (561,731; http://www.ncbi.nlm.nih.gov/dbEST), EST-SSRs have become an attractive alternative to complement existing SSR collections, and 101 new EST-SSRs loci from bread wheat have recently been added to the wheat genetic map (Gao et al. 2004). Comparisons between genomic-SSRs and EST-SSRs have revealed that wheat EST-SSR markers have a lower level of polymorphism but produce higher quality patterns (Eujayl et al. 2001, 2002; Leigh et al. 2003). The genetic diversity has also been assessed in a collection of 52 elite exotic wheat genotypes (Gupta et al. 2003), and the results suggest that EST-SSRs can be successfully used for a variety of purposes and may be superior to genomic SSRs for diversity estimation.

In addition to the advantages of genomic SSR markers mentioned earlier, EST-SSRs show a high level of transferability to closely related species because they originate from conserved transcribed regions that are better conserved between the genomes; this consequently facilitates their use in comparative mapping (Yu et al. 2004a), as do restriction fragment length polymorphism (RFLP) markers derived from cDNA (for a review, see Gupta and Rustgi, 2004). The transferability of bread wheat EST-SSRs across 18 wild relatives and five cereal species (barley, rye, oat, rice and maize) was recently studied with 78 EST-SSR markers (Gupta et al. 2003). More than 80% of cross-species transferability was observed with wild relatives; this rose to as high as 90% with at least one of the cereal species. Similarly, a relatively high level of transferability (55%) of EST-SSRs was found from barley to wheat (Holton et al. 2002). In another study, 368 EST-SSRs derived from five different grass species (barley, maize, rice, sorghum and wheat) were developed and 149 loci integrated into a reference wheat genetic map; 80 of these were subsequently assigned to chromosomes using nullisomic-tetrasomic lines (Yu et al. 2004b).

The purpose of the project reported here was to investigate 300 primer pairs designed from bread wheat ESTs containing at least one SSR and to study their transferability to durum wheat and other related diploid species carrying the A, B and D genomes as well as rye, *Agropyron*, rice and barley. Their chromosomal assignment was assessed and compared to the one obtained by in silico mapping on the rice pseudo-molecule. Homology relationships with barley and rice ESTs and the evolution of SSRs within ESTs are also discussed.

Materials and methods

Primer design and SSR amplification and detection

SSRs were detected among the 46,510 Génoplante EST contigs (version 1 02/2002) following the method of Nicot et al. (2004). A microsatellite was defined as a sequence containing a minimum of three repeats of a motif comprising from one to six nucleotides, with a total length of at least 12 nucleotides. Primers were designed using PRIMER software (version 0.5, Whitehead Institute for Biomedical Research, Cambridge, Mass.) based on the following criteria: (1) primer length ranging from 18 to 22 bp with 20 bp as the optimum; (2) product size ranging from 100 to 400 bp; (3) melting temperature (T_m) between 57°C and 63°C with 60°C as the optimum; (4) a GC% content between 20% and 80%; (5) maximum acceptable primer self-complementarity of five bases; (6) maximum acceptable 3′ end primer self-complementarity of three bases. Primers were selected on the basis of their containing as few as possible repeated sequences and with the 3′-end of the two primers ending with a C or G when possible. Primer sequences were subjected to BLAST analysis against an in-house database to avoid redundancy with those that already exist. On this basis, 300 primer pairs were selected and designated as CFE (primer sequences available on Graingenes: http://wheat.pw.usda.gov/index.shtml). Each forward primer was M13-tailed [M13: 5′-CACGACGTTGTAAAACGAC-3′, synthesis MWG (Germany)]. PCR analyses using the M13 protocol were performed twice as described by Nicot et al. (2004) with an annealing temperature of 60°C for 30 cycles (30 s 94°C, 30 s 60°C, 30 s 72°C) and 56°C for eight cycles. Polymorphisms were visualised using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif.).

Plant material

Eight hexaploid wheat lines corresponding to the parents of five mapping populations were used for polymorphism screening of the microsatellites: W7984 (synthetic wheat; Van Deynze et al. 1995) and cultivars Opata, Courtot, Chinese Spring, Eurêka, Renan, Arche and Récital. Eight other species were used to study the transferability of EST-SSRs: *Triticum durum* cv. Bidi 17, *T. monococcum* accession 68212, *Aegilops speltoides* accession 37, *Ae. tauschii* accession 15, rye cv. Dankovski Nove (*Secale cereale*), barley cv. Plaisant

(*Hordeum vulgare*), *Agropyron elongatum*, and rice cv. IR64 (*Oryza sativa*). A set of 20 nulli-tetrasomic (NT) and three ditelosomic (DT) wheat lines (kindly provided by Dr. Steve Reader, John Innes Centre, UK) was used for chromosomal assignment of the markers. DNA extraction was performed from fresh leaves using a CTAB protocol as previously described (Tixier et al. 1998).

Sequence analysis and in silico mapping

To assign putative functions to ESTs containing microsatellites, we compared the sequences of the ESTs to the SwissProt and TrEMBL protein databases using BLASTX algorithms (Altschul et al. 1990), with expected value of 1 e^{-5} as a significant homology threshold. Putative functions were attributed according to the definitions given at http://www.godatabase.org/cgi-bin/amigo/go.cgi. TBLASTX searches were also performed against rice and barley NCBI unigene sets (National Center for Biotechnology Information, Washington, D.C.; http://www.ncbi.nlm.nih.gov/) to study the degree of conservation of the repeated motif between the three species. In addition, the EST sequences were compared to the rice pseudo-molecules (http://www.tigr.org/tdb/e2k1/osa1/) using TBLASTX and BLASTN to identify putative orthologues on rice chromosomes. *E*-Values of less than 1 e^{-5}, 1 e^{-10}, 1 e^{-25}, 1 e^{-50} and 1 e^{-100} were selected. Potential bias in the distribution of wheat EST orthologues on rice pseudomolecules was investigated using the relative error (E_r). This value was computed as follows: the rice gene proportion for each chromosome was calculated as the ratio between the number of genes on the rice chromosome and the total number of genes on the rice genome (values at http://www.tigr.org/tdb/e2k1/osa1/). The expected number of hits was then evaluated as the product of the number of wheat EST blasted (251) by the rice gene proportion. Observed hit values were compared to the expected values using a classical binomial test from SPLUS, and only significant values at the 0.05 or 0.01 threshold were retained.

Results

Amplification of EST-SSRs in wheat

The 300 primer pairs were selected among a collection of 46,510 wheat ESTs and were tested on genomic DNA of eight reference hexaploid wheat cultivars in order to assess amplification and polymorphism; 80% (240/300) of these led to an amplification product. In some cases, differences were observed between the expected and the observed size of the amplification product. Among the 240 primer pairs, 51 (21.3%) gave an amplification product either larger than expected, suggesting the presence of an intron in the genomic sequence, or smaller, suggesting the occurrence of deletions within the genomic sequences or a lack of specificity which may lead to amplification of another copy of a gene family.

In general, the EST-SSR markers produced high-quality patterns (Fig. 1). Overall, 198 markers—representing about 80% of total number of primer pairs giving an amplification (240)—produced strong and clear bands; 120 primer pairs gave a single amplification product on wheat, while 120 gave complex patterns with more than one band. Among the latter group, 38 gave three bands likely corresponding to the three homoeologous (A, B, D) copies of the gene. As expected, single-copy EST-SSRs presented a lower level of polymorphism in bread wheat (56%) than multiple-copy (3) EST-SSRs (79%).

Assignment to wheat chromosomes

The 240 primer pairs that yielded products were used for amplification on 20 NT and three DT (to balance the missing NT) lines derived from Chinese Spring. Overall, 177 primer pairs gave rise to PCR products that could be assigned to wheat chromosomes. The remainder (63) of the PCR products could not be assigned because either the profile produced was too indistinct or too complex (42) or because the primer pair amplified a product in all of the NT and DT lines (21). Among the 177 primer pairs, 99

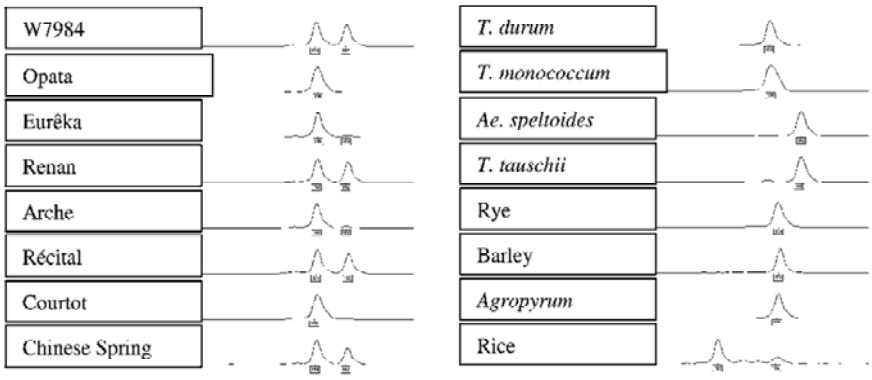

Fig. 1 Example of a high-quality pattern obtained on the ABI 3100 capillary system after M13 PCR amplification of the EST-SSR CFE154 on the eight wheat varieties (*left*) and the eight related species (*right*)

Table 1 Distribution of EST-SSR loci according to their assignment to wheat chromosomes and homoeologous groups

	Homoeologous group							
	1	2	3	4	5	6	7	Total
Chromosome A	14	9	16	17	16	17	12	101
Chromosome B	16	15	15	10	13	14	14	97
Chromosome D	8	12	19	15	15	11	16	96
Total	38	36	50	42	44	42	42	294

were assigned to a single locus, while 78 others were assigned to a maximum of four loci. Most of these latter (70) were found at loci located on the same homoeologous group. Thus, 294 EST-SSR loci distributed across all 21 wheat chromosomes (Table 1) were assigned, with 101, 97 and 96 loci on the A, B and D genomes, respectively. No significant bias in the chromosome or in the genome location was observed, and the distribution ranged from 8 (on chromosome 1D) to 19 loci (3D).

Transferability of wheat EST-SSRs to closely related species

The 300 primer pairs were used on genomic DNA of *T. durum*, *T. monococcum*, *Ae. speltoides* and *Ae. tauschii* accessions. As expected, a high transferability to *T. durum* (AABB; 90.4%) was found. Even the EST-SSRs loci assigned to the D genome showed a good transferability (93.7%). Transferability to the three diploid species carrying the A (*T. monococcum*), B (*Ae. speltoides*) and D (*Ae. tauschii*) genomes was also very good (85.3%, 79.2% and 76.7%, respectively).

The relationships between the copy number in wheat, the degree of polymorphism and the transferability to diploid genomes were also investigated (Table 2). Among the 240 primer pairs giving an amplification product on wheat, 98 were selected and classified into two sets based on the number of bands revealed and their chromosomal location: one set consisted of primer pairs giving a single amplification product (69) that corresponded to one locus, while the other set produced three amplification products (29), which corresponded to the three homoeologous copies. All multiple-copy as well as single-copy markers gave an amplification product on either *T. durum* or on wheat diploid species carrying the A, B and D genomes, indicating a high level of gene-sequence similarity between these species and wheat and, thus, a high level of transferability.

The relationship between the genomic location of the EST-SSR loci on the A, B or D genomes of bread wheat and the transferability to the three diploid ancestral genomes was assessed. Most of the 101 EST-SSR loci assigned to the A genome of bread wheat amplified the very least on *T. monococcum* (97%, Table 3). Only two primer pairs were specific for the B and D diploid genomes and one was specific for the A hexaploid genome. More than 70% amplified on all three diploid genomes, while about 9% amplified on the A and B genomes and 4% on the A and D genomes. The remaining 11% were specific for the A diploid genome. All except one of the 96 EST-SSR loci assigned to the D genome of hexaploid wheat amplified on *Ae. tauschii* (99%); most (85.4%) amplified on the three diploid genomes, while only 5.2% amplified on both the D and A genomes and 6.3% on both the D and B genomes. Only 2.1% remained specific to the D diploid genome. On the contrary, 84% of the 97 loci assigned to the B genome of hexaploid wheat amplified on *Ae. speltoides*, and only 1% were specific to the B diploid genome; 9.3% amplified on both the B and A genomes, 1% on the B and D genomes, while 73.2% amplified on all three genomes. Interestingly, 5% were specific for the hexaploid genome, while 6% amplified on both the A and D diploids and 4% on the A genome species only. Thus, our results show a better conservation between the A and D genomes of bread wheat and the corresponding genomes of the diploid species than between the B genome of bread wheat and the B genome of *Ae. speltoides*, the latter supposedly being the closest species to the ancestral donor.

Transferability of wheat EST-SSRs to other grass species

We used the same set of 98 EST-SSRs giving a single amplification product (69) that corresponded to one

Table 2 Relationships[a] between the number of bands revealed by EST-SSRs and the results of amplification and polymorphism

Number of bands	Polymorphism in common wheat		Transferability to *Triticum monococcum*, *Aegilops speltoides*, *Ae. tauschii*, *T. durum*		Transferability to rye, barley, *Agropyrum* or rice	
1 band	+	39	+	39	+	29
					–	10
	–	30	+	30	+	23
					–	7
3 bands	+	23	+	23	+	23
	–	6	+	6	+	6

[a] +, Indicates either polymorphism was observed on at least one of the eight wheat varieties studied or amplification on at least one related species; –, indicates either the absence of polymorphism on the eight wheat varieties or the absence of amplification on all related species

Table 3 Transferability of EST-SSRs to the A, B and D diploid species as a function of their chromosomal location

Hexaploid genome[a]	ABD	AB	AD	BD	A	B	D	NA	Total
A genome	74 (73.3%)	9 (8.9%)	4 (4%)	2	11 (10.9%)			1	101
B genome	71 (73.2%)	9 (9.3%)	6	1 (1%)	4	1 (1%)		5	97
D genome	82 (85.4%)	1	5 (5.2%)	6 (6.3%)			2 (2.1%)		96

[a]ABD, Amplification in all the three diploid species; AB, AD, BD, amplification in A and B, A and D, and B and D diploid species, respectively; A, B, D, amplification, respectively, in the A, B and D diploid species only; , no amplification

locus or to three amplification products (29) that corresponded to the three homoeologous copies described previously to study the transferability to related grass species. Overall, 67.9%, 50.4% and 55.8% of the markers gave an amplification product in rye, barley and *Agropyrum*, respectively. The transferability to rice was lower, accounting for 28.3%. About 74% of the single-copy SSRs (Table 2) showed an amplification on at least one of the related species tested (rye, barley, *Agropyrum* or rice). Among these, those located on the A and D genomes were the most transferable (81.5% and 81%, respectively), while those from the B genome exhibited a lower level of transferability (61.9%). All of the multiple-copy SSRs gave an amplification product on at least one of the species. This confirmed that the primers producing numerous bands are less specific than those giving single amplification products and, consequently, are more transferable.

In order to study the relationships between genomic location and transferability between wheat and rice, we investigated on which rice chromosomal regions (telomeric or centromeric) homologues of the wheat EST-SSRs were located. The 265 EST-SSR sequences were BLASTed against the 12 rice pseudomolecules with threshold *E*-values of $1\ e^{-5}$, $1\ e^{-10}$, $1\ e^{-25}$, $1\ e^{-50}$ and $1\ e^{-100}$ (http://www.tigr.org/tdb/e2k1/osa1/pseudomolecules). The results are illustrated in Fig. 2 and Table 4. They show that the rice homologues were not uniformly distributed on the whole rice genome (Table 4), despite the fact that the wheat EST-SSRs were randomly chosen. Rice chromosomes 2 and 3 were significantly overrepresented (E_r = 40.6%, and 50.3%; significant at $P = 0.05$ and 0.01, respectively), while chromosome 11 was significantly underrepresented ($E_r = 65.9\%$; significant at $P = 0.01$). In addition, the wheat ESTs corresponding to those located on rice chromosome 3 showed a better similarity, indicating a higher level of conservation between rice and wheat genes in this region. Interestingly, the wheat EST-SSRs which were transferable to rice were mainly located in telomeric regions (Fig. 2) where the highest sequence similarity was found, indicating a clear relationship between the degree of sequence conservation and the level of transferability. A significant bias at $P = 0.05$ was observed on rice chromosome 5 (Table 4), indicating that the distal part of the long arm of this chromosome was best conserved between wheat and rice at the level of the flanking sequences of the SSRs.

Relationship between the putative function of the ESTs and the level of transferability to rice

The 265 ESTs containing a SSR were BLASTed against the SwissProt and TrEMBL protein databases in order to identify their putative functions. Using an *E*-value of $1\ e^{-5}$ as the threshold, 168 (63.4%) EST sequences had one hit with one sequence from SwissProt, while 228 (86%) had one hit with the TrEMBL database. We classified the 168 EST sequences that gave a hit on the SwissProt database according to the criteria given on the gene ontology database (AmiGO, http://www.godatabase.org/cgi-bin/amigo/go.cgi). Three different classes of function are proposed on this basis: (1) biological processes, which means phenomena marked by changes that lead to a particular result; (2) cellular components, including gene products that are parts of macromolecular complexes; (3) molecular function, which means elemental activities, such as catalysis or binding. Most of the ESTs (107, 63.7%) were involved in a molecular function, while 23 (13.7%) played a role in biological processes and 19 (11.3%) were cellular components (Fig. 3). The 19 remaining ESTs had no putative function. With respect to those with a molecular function, almost 50% of them (53) were involved in catalytic activity, about 15% in binding activity, and some in transporter (8) or chaperone activity (8). This distribution corresponds to the one observed on the whole EST library (about 170,000 sequences, data not shown), indicating a random distribution of the EST-SSRs among the different classes of genes.

To study further the relationship between transferability to rice and EST function, we selected the 69 EST-SSRs giving an amplification product on rice and examined their putative function. Among the 44 showing a hit to the SwissProt database, similar proportions were observed between the three classes of function, indicating no significant bias between gene function and the level of transferability to rice.

With respect to gene location, 123 of the 168 EST-SSRs sequences found in SwissProt occurred inside open reading frames (ORFs), while only 45 were in non-coding regions (41 in 3′-untranslated regions (UTRs) and 4 in 5′-UTRs), suggesting that SSRs occurred more frequently in ORFs than in untranscribed regions. The proportions of di-, tetra- and pentanucleotides that cannot change without mutational consequences inside the ORFs were not different from those observed for the whole library (Nicot et al. 2004).

682

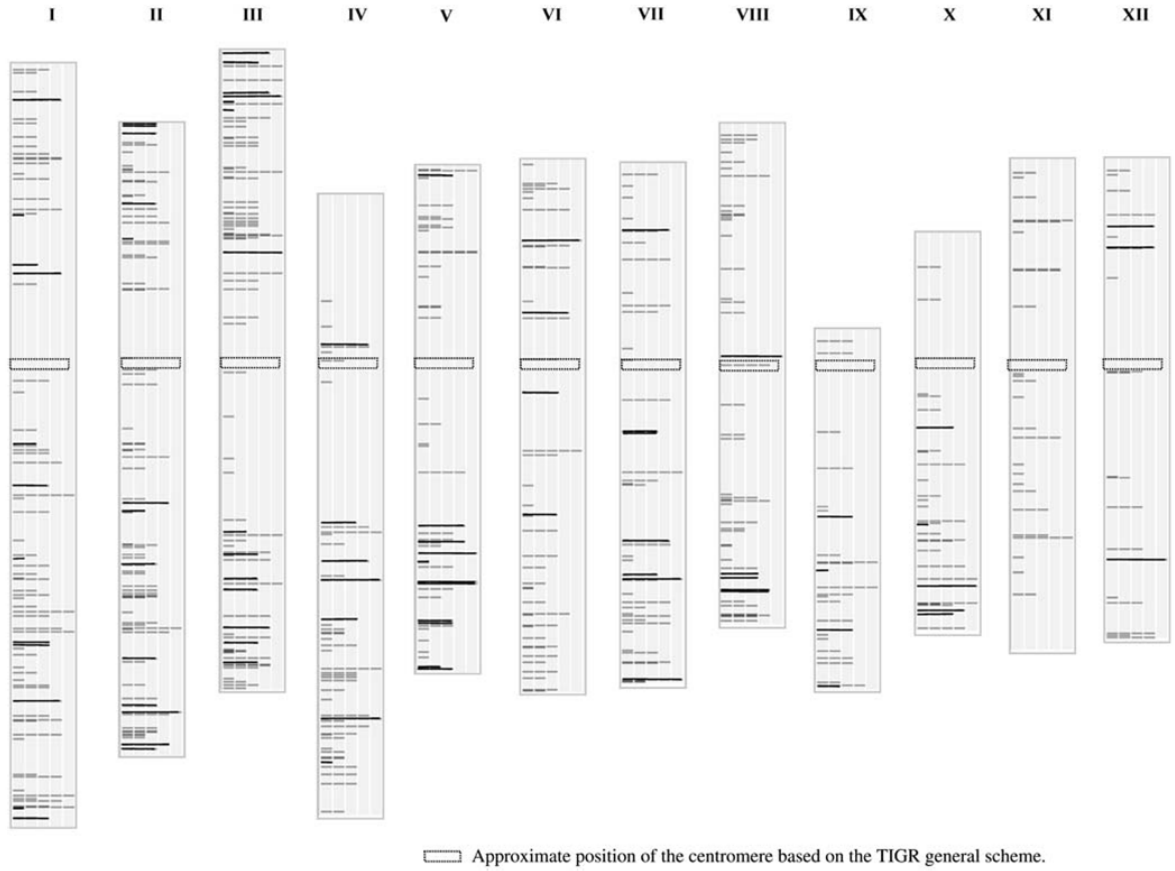

Fig. 2 Results of the BLAST analysis of the wheat EST-SSRs on the 12 rice pseudo-molecules. Five different expected values were selected: 1 e ^{5}, 1 e ^{10}, 1 e ^{25}, 1 e ^{50} and 1 e ^{100}. Approximate position of the hit for each locus is indicated with *solid bars* according to an e-value for one bar of 10 ^{5} up to an e-value for five bars of 10 ^{100}. The EST-SSRs that were transferable to rice are indicated by *black bars* and all others are indicated by *grey bars*. Approximate position of the centromere is indicated with a *dotted rectangle*

Conservation of the SSR motif between wheat, barley and rice EST-SSRs

Wheat EST sequences were BLASTed against rice and barley EST unigene sets (http://www.ncbi.nlm.nih.gov/) in order to determine if a repeated motif existed within homologous rice and barley sequences. Of the 265 EST

Table 4 Distribution of 251 wheat EST loci on the 12 rice pseudomolecules

	Rice pseudomolecules												
	1	2	3	4	5	6	7	8	9	10	11	12	Total
Chromosome length (%)	11.8	9.7	9.9	9.6	7.9	8.2	8.1	7.8	5.8	6.2	7.6	7.4	100
Expected gene proportion[a] (%)	12.9	10.2	11.4	8.9	7.9	8.2	8.1	7.3	5.4	6.2	7.0	6.5	100
Expected no. of genes[b]	32.4	25.6	28.6	22.3	19.8	20.6	20.3	18.3	13.6	15.6	17.6	16.3	251
Observed no. of genes[c]	36	36	43	21	20	21	19	15	12	12	6	10	251
E_r[d] (%)	11.1	40.6*	50.3**	5.8	1.0	1.9	6.4	18.0	11.8	23.1	65.9**	38.7	
Expected Transferability[e]	14.1	14.1	16.8	8.2	7.8	8.2	7.4	5.8	4.7	4.7	2.3	3.9	98
Observed Transferability[f]	14	14	15	9	13*	5	8	6	4	6	1	3	98

*, **Significant at the 0.05 and 0.01 levels, respectively
[a]Expected gene proportion, Number of genes on the rice chromosome/total Nb of genes on the rice genome
[b]Expected number of genes, number of rice homologue loci with a hit with wheat EST (251) × expected gene proportion
[c]Observed number of genes, Number of hits observed on each rice pseudomolecule
[d]Er (relative error) = expected value − observed value / expected value × 100 (%)
[e]Expected transferability, Expected transferability between wheat and rice = [observed no. of genes × no. of transferable homologue loci (98)]/no. of rice homologue loci with a hit with wheat EST (251)
[f]Observed transferability, Observed transferability between wheat and rice

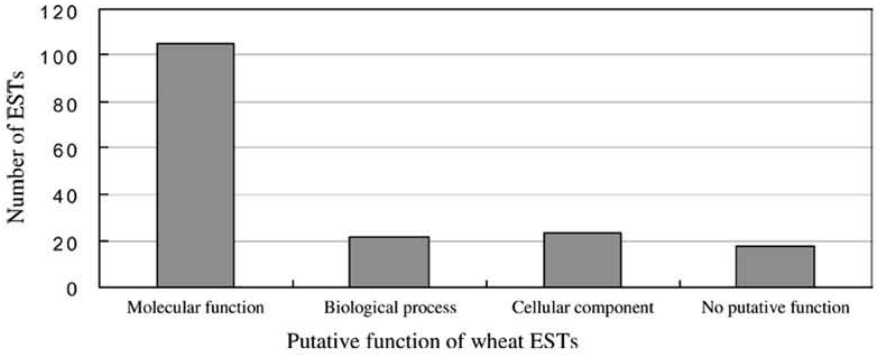

Fig. 3 Putative function annotation of ESTs containing microsatellites according to the criteria given on the gene onthology database (AmiGO, http://www.godatabase.org/cgi-bin/amigo/go.cgi)

sequences, 236 had at least one hit to rice ESTs, and 228 had a hit to barley ESTs using an *E*-value of 1 e^{-5}. Fifty-four ESTs showing a hit to both barley and rice EST databases and having large SSR-flanking regions were studied in detail (Table 5). Among these, 27 amplified in wheat, barley and rice simultaneously, 11 in both wheat and barley, three in both wheat and rice and 13 in wheat only. For those amplifying in the three species, most of the sequences (24/27 for barley, 20/27 for rice) presented similar repeated motifs (perfect or non-perfect). An example is given in Fig. 4a. For EST-SSR CFE1, a trinucleotide motif $(cgc)_4$ was observed in wheat. The same motif was also present in barley and rice, but it was repeated five times in the former and two times in the latter. Repeats were not present in three sequences in barley and seven in rice, with two of the barley sequences being common with the seven rice sequences. For the EST-SSRs amplifying in wheat and barley only, 9 of the 11 presented a similar repeated motif in barley, while no repeats were present for the two others. For those amplifying in wheat and rice only, a repeated motif was always detected in the rice sequences. For the EST-SSR CFE59 (Fig. 4b), the motif was different in wheat and barley (ccgt) from rice (cgc),

Table 5 Degree of conservation of the SSR motifs in rice and barley EST sequences

Amplification in	Number of markers	Repeated motif in barley				Repeated motif in rice			
		Existence			No existence	Existence			No existence
		Perfect	Non-perfect	Different		Perfect	Non-perfect	Different	
Wheat, barley and rice	27	15	9	0	3	9	11	0	7
Wheat and barley	11	5	4	0	2	2	3	0	6
Wheat and rice	3	1	0	1	1	0	2	1	0
Wheat	13	4	2	0	7	1	1	0	11
Total	54	24	15	1	13	12	17	1	24

Fig. 4 Sequence comparison between wheat, barley and rice EST sequences for the EST-SSRs CFE1 (**a**), CFE59 (**b**) and CFE43 (**c**). In each case, the SSR is indicated by *boxed bold characters*. Identical nucleotides are related with a *double dot*

suggesting a common origin for the two triticeae species and a different one for rice. For EST-SSR CFE43 (Fig. 4c), the motif was composite in rice $[(cct)_2(ccg)_3]$, while it was unique but different in wheat $[(cct)_4]$ and barley $[(ccg)_4]$, suggesting that a motif similar to the one present in rice was present in the common ancestor and then evolved differently after the divergence of the three species. For the EST-SSRs amplifying in wheat only, repeats did not exist in the corresponding region for most of those for rice (11/13) and, to a lower extent, for barley (7/13). In some cases, the repeated motifs did exist in one or in both barley and rice species, but no amplification occurred. This is likely due to mispairing at the primer level because of the presence of mutations in the flanking regions. One possible explanation for the absence of repeated motifs is that the SSRs arose after the divergence between wheat and rice or wheat and barley. However, we cannot exclude the possibility that we are not comparing orthologues but paralogues of the genes that followed different evolutionary histories.

Discussion

In our study, 80% of the primer pairs successfully amplified EST-SSR products, and over 80% of these produced strong and clear profiles in wheat. About 64% of the primer pairs yielded fragments of the expected size; this is in sharp contrast to results obtained with genomic SSRs, for which only 36% yielded fragments of the expected size, with many resulting in a smear (Röder et al. 1995). This results confirms that EST-SSRs give better profiles than genomic SSRs (Eujayl et al. 2001, 2002; Leigh et al. 2003).

With respect to chromosomal assignment, 177 EST-SSRs revealed 291 loci that were randomly distributed along all the 21 wheat chromosomes, indicating no prevalence for the location of EST-SSRs. Up to 45% of the EST-SSRs identified more than one locus, suggesting an amplification of either the homoeologous or homologous copies. Some bands were not assigned because the products were amplified in all NT and DT lines, indicating that these EST-SSRs produced co-migrating bands of the same size for more than one copy of the gene. If necessary, such markers can be converted in locus-specific markers by cloning and sequencing each amplification product, identifying specific mutations by comparing the sequences of the different gene copies and designing new pairs of primers specific for each copy. Such specific EST-SSRs would represent excellent markers for phylogenetic studies.

EST-SSR markers are more transferable across closely related genera than genomic SSRs because they originate from the coding regions which have a higher level of sequence conservation than intergenic regions. Our results confirmed that wheat EST-SSRs show high simultaneous transferability (86.6%) to at least two of the A-, B- and D-related diploid genomes, while fewer than 15% were only specifically transferable to one genome. These results contrast with those observed with genomic SSRs which are more genome-specific and thus less transferable to related species (Sourdille et al. 2001). Similar results were reported in soybean where genomic SSRs are mostly restricted to congenic species (Peakall et al. 1998). In addition, our results show that EST-SSRs in wheat may be used to detect the three homoeologous copies at the same time, as confirmed by the chromosomal assignment of EST-SSR loci using the set of Chinese Spring compensating nullisomic-tetrasomic lines (Sears 1966).

The high transferability to rye (67.9%) that we observed contrasted with the low transferability of wheat genomic SSRs to rye reported by Kuleung et al. (17%; 2003) and by Röder et al. (6.75%; 1995). Transferability to barley (55.8%) and rice (28.3%) was also relatively good under our conditions. Our results with barley were in total agreement with those of Yu et al. (2004a), who reported 53% of transferability between these two species. However, the same authors mentioned a higher level of transferability to rice (45%). This was due to the selection of primers that was done in well-conserved regions between wheat and rice, while our primers were designed on the wheat EST sequences only. Our results can thus be improved by using homology information from rice and barley databases, and they largely confirmed the higher power of EST-SSRs compared to genomic SSRs for transferability studies (Eujayl et al. 2003; Gupta et al. 2003; Thiel et al. 2003).

The analysis of the relationships between the level of transferability and the number of bands revealed that EST-SSRs showing three bands had a higher transferability than those with only one band, even to more distantly related species such as barley, rye and rice. This is in accordance with the results reported by Guyomarc'h et al (2002a), who studied genomic SSRs developed from *Ae. tauschii*. The single-band markers may amplify more conserved regions, which would explain the fact that most of them yielded one product compared to multiple-banded markers. This suggests that EST-SSR markers giving three bands are more useful for comparative mapping.

Recent comparisons of cytogenetic maps with genetic linkage maps based on chromosome deletion lines have revealed the existence of gene-rich regions in the wheat genome, most of which are located in the distal parts of the chromosomes (Werner et al. 1992; Gill et al 1996; Faris et al. 2000; Weng et al. 2000; Sandhu et al. 2001, Sourdille et al. 2004). BLASTing the 265 wheat ESTs against rice's pseudo-molecules showed that despite the fact that EST-SSRs were randomly chosen, the distribution was uneven. Chromosomes 2 and 3 from rice were overrepresented while chromosome 11 was underrepresented. The results also showed that our set of wheat ESTs had a better sequence similarity with genes on rice chromosome 3 compared to the others. This suggests that in the course of evolution, a relatively higher level of gene conservation was maintained between rice chromosome 3 and the corresponding

homoeologous group 4 in wheat (La Rota and Sorrells 2004; Yu et al. 2004b). This may partly explain the lack of polymorphism that is frequently observed on homoeologous group 4, especially on chromosome 4D (Nelson et al. 1995; Cadalen et al. 1997; Paillard et al. 2003). EST-SSRs located on wheat group 4 chromosomes would be more effective for comparative mapping studies in the future.

In silico analysis demonstrated that 73.2% of the EST-SSRs were located in ORFs in wheat, which was similar to observations made on other plants (Gupta et al. 2003). Many studies have shown that SSRs are prevalently found in 5′-UTR (Cho et al. 2000; Scott et al. 2000; Gupta et al. 2003; Thiel et al. 2003; Yu et al. 2004a). In contrast, our results showed that SSRs were more often located in 3′-UTRs than in 5′-UTRs. This is due to a bias in our sampling of EST-SSRs, which mainly originate from sequences of 3′-end cDNAs (Génoplante collection) and also from the fact that 5′-UTRs are less frequent in the databases since they are more difficult to obtain. We were not able to correlate the presence of a SSR in a coding sequence to a specific cellular function. SSRs seem to be randomly distributed among gene classes since the EST-SSRs' function distribution was consistent with the distribution of gene function in the EST libraries.

We further investigated 54 wheat ESTs which were found in both barley and rice databases and were long enough for significant BLAST analysis. A distinct correlation was found between the rate of success of amplification in all three species (wheat, barley and rice) and the degree of conservation between the repeated motifs (89% and 74% for barley and rice, respectively). However, the PCR yield was sometimes weaker than in wheat, suggesting that flanking sequences were subjected to mutations at one or several bases. More wheat repeated motifs remained identical in barley than in rice. This was expected since wheat ESTs show a higher degree of homology with barley ESTs than with rice ESTs as the former diverged from each other more recently during evolution (10 mya between wheat and barley compared to 50 mya between wheat and rice). Most of the conserved motifs between the three species were trinucleotides (77%), which is in agreement with the size distribution of the motif among ESTs (Nicot et al. 2004). Interestingly, one-half of the conserved motifs were (ccg), and all of them contained at least one g (or one c). When the SSR motifs were identical between the three species, no significant difference was observed with respect to the number of repeats even if rice had frequently fewer repeats, suggesting that the evolution rate of the EST-SSRs could be slower in rice than in wheat and barley.

In some cases, repeated motifs exist at a proto-microsatellite state in rice; this also occurs on occasion in barley. In these cases, our results suggest that the wheat microsatellites evolved following the divergence between the Bambusoideae and the Pooideae, or even later. For some of the motifs that are proto-microsatellites in rice but appeared as microsatellites in barley, the differentiation can be refined at the level of the Triticodeae. Several types of mutation may have transformed proto-microsatellites into microsatellites: point mutations, frameshift mutations or the transposition of mobile elements. The frequency of such events is not different between the three species. If the proto-microsatellites have not evolved in rice, this may be due by chance or be associated with selection pressure. Since wheat is a polyploid which bears most of the genes in triplicate, the constraint is probably lower and proto-microsatellites can evolve faster than in rice or barley.

Finally, two hypotheses can be raised for those motifs that are absent from rice and barley. The first one is that they could have arisen in the *Triticum* lineage only. Such a hypothesis can be confirmed by testing a large number of additional grass species in order to identify the divergence points for these EST-SSRs. This could be a valuable contribution to the reorganisation of the phylogeny of the grass species. We would also be able to date some of the differentiations using strategies such as the one described in Thuillet et al. (2002), who estimated the mutation rate of ten SSR loci in durum wheat. Such a programme is currently being pursued in our laboratory. The second hypothesis is that the wheat and rice ESTs that are compared are not true orthologues. Some of the ESTs we used had several hits on the rice pseudomolecules, and it is possible that only one or few of them have the SSR, whereas the one expressed and found in the databases does not carry the microsatellite.

Thus, we can conclude that wheat EST-SSR markers show a high transferability across a large range of species. This transferability make them a powerful tool to work on orphan wild species such as *Agropyrum* where less effort has been expended to develop genomic resources such as molecular markers. These species are a very important source of both abiotic and biotic resistance genes (Doussinault et al. 1983), and molecular markers are precious tools to use and reduce the introgressions of genes (location, size of the introgression) from these species. EST-SSRs are thus excellent molecular markers that can now be used extensively in breeding programmes by MAS methods.

Acknowledgements The authors thank gratefully G. Gay and A. Loussert for growing the plants and S. Reader for providing the aneuploid lines. Gilles Charmet is also acknowledged for the statistical analyses. This work was funded by the China Scholarship Council (CSC). All experiments comply with the current laws of France.

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2.2 Wheat EST-SSRs for tracing chromosome segments from a wide range of grass specie (Plant Breeding, accepted)

Wheat EST-SSRs for tracing chromosome segments from a wide range of grass species

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With: - 2 tables
- 2 supplementary data files

Abstract

Transferability of 116 common wheat EST-SSR markers was investigated on 158 accessions representing 18 grass species to identify new alleles useful for wheat improvement. Transferability among the Triticeae ranged from 73.7% for *Ae. longissima* to 100% for wheat sub species (*T. compactum*) but was also good for less related species such as rye (72.8%) or maize (40.4%). On average, the number of alleles/locus detected by EST-SSR markers was 3.1 on hexaploid wheat. The PIC values simultaneously estimated for *T. aestivum* and *T. durum* were similar for the two species (0.40 and 0.39 respectively). The allelic diversity within allogamous species was higher (0.352-0.423) compare to that of *T. aestivum* and *T. durum* (0.108 and 0.093 respectively). *T. aestivum* and *T. durum* shared the largest number of alleles (74.6%) while among the three ancestral diploid species of bread wheat, *Ae. tauschii* had the highest percentage of common alleles with *T. aestivum* (57.4%). These results indicate that grasses orphan species can be studied using wheat EST-SSRs and can serve as a source of new alleles for wheat genetic improvement.

Key words

Triticum – microsatellites – bread wheat – genetic diversity – markers – relatives

Introduction

Significant advances in the understanding of the plant biology as well as in the management and exploitation of genetic resources must be achieved to face increasing human needs. Concerning genetic resources, genetic variability in grasses and especially in wheat (*Triticum aestivum* L.) is extremely extended but remains largely underexploited. This is primarily due to the poor knowledge of the capacities of these resources in terms of biotic and abiotic resistances as well as their potential for quality or yield performances. They are also bearing many traits of low agronomical interest such as large plant height, lodging sensitivity, free threshing and hulled kernels which make the breeders reluctant to use them because of the linkage drag of these negative traits. Moreover, these species are often difficult to cross with wheat because of the presence of *Kr* genes (Lein 1943; Snape et al. 1979; Falk and Kasha 1981). Only few recombinations are established between wheat and alien chromosomes and it is almost impossible to evaluate the quantity of foreign DNA introduced. We can suspect that the bad crossability and the low recombination will soon be overcome with the positional cloning of the genes involved in these processes (*Ph1*, *Kr*, *SKr*; G Moore, personal communication; M Bernard and C Feuillet, unpublished results). However, the linkage drag and the evaluation of the sizes of the introgressions still remain a problem since these phenomena depends on the power of the evaluation of these latter and thus of the genotyping capacities available. It was demonstrated that cytogenetically controlled introgressions of alien fragments of chromosomes in wheat may have a positive effect on resistance to various traits as well as for yield or bread making quality (Jarwal et al. 1996; Singh et al. 1998; Villareal et al. 1995, 1998; Hoisington et al. 1999; Kim et al. 2003; Reynolds et al. 2001, 2005).

At the present time, low attentions have been paid on the study of wheat related species especially in genomic areas while genomics has exploded in wheat in the last five years leading to better marker-assisted selection and to the positional cloning of a number of genes of agronomic interest. The genomic efforts should primarily seek on the development of molecular markers because they are easy to produce and to use and because they are involved in all the further analyses including QTL detection, positional cloning and diversity studies. In polyploid wheats, microsatellites also referred to as Simple Tandem Repeats (SSRs) constitute the marker of choice because of their co-dominant inheritance, their even distribution along the genome, their high polymorphism level, their good reproducibility and their genome specificity (Plaschke et

al.1995; Röder et al. 1995, 1998; Bryan et al. 1997; Guyomarc'h et al. 2002a, b; Nicot et al. 2004). However, they are expensive to develop because it is needed to create and screen enriched libraries, to sequence a lot of clones and to test numerous pairs of primers prior to have a useful marker. In addition, because of their genome specificity, genomic SSRs developed from wheat cannot be used on related species (Sourdille et al. 2001).

A new type of SSRs was recently developed from the large wheat EST collections (599,989 ESTs; http://www.ncbi.nlm.nih.gov/dbEST; EST-SSRs) which were developed in the past five years. Compared to the genomic SSRs derived from enriched genomic DNA libraries (g-SSRs), EST-SSRs give better profiles (Holton et al. 2002; Gupta et al. 2003) and recently, Zhang et al. (2005a) demonstrated that bread wheat EST-SSRs showed a high level of transferability to close and wild relatives of wheat because they are mainly derived from conserved coding regions. They can thus be used as a starting point for further genomic studies on wheat-related orphan species such as *Aegilops*, *Agropyron*, *Enhardia*, *Elymus*, *Thynopyron* or other *Triticum* species. Some of these species can be used to derived synthetic wheats which have already been shown to possess a high genetic diversity (Lage et al, 2003; Zhang et al, 2005b) as well as favorable qualitative (Kema et al, 1995; Ma et al. 1995; Lage et al, 2001; Mujeeb-Kazi et al. 2001) and quantitative traits (Villareal et al. 2001).

In this paper, we report the results of the genetic study of 168 accessions from 18 different species from the grass family representing 17 different genomes using a set of 116 EST-SSRs that we recently developed (Zhang et al., 2005a). The objectives of this study were: (1) to evaluate the transferability of the EST-SSRs to these species; (2) to characterize the allelic diversity on a set of accessions for each species; and (3) estimate the EST-SSR PIC values within *T. aestivum* and *T. durum* species.

Materials and Methods

Plant Material

A total of 168 accessions, representing 18 grass species and 17 genomes of the *Triticeae* tribe were used (see electronically supplementary data). This included diploid and polyploid species and autogamous as well as allogamous species. Between two and six accessions for each species were randomly chosen among our collection except for *T. aestivum* and *T. durum* where respectively 22 and 25 varieties were selected. Seeds were

mainly obtained from the Centre of Biological Resources on Cereal Crops (INRA-Clermont-Ferrand), and from Jacques David (INRA Montpellier, tetraploid and *Aegilops* species), and Philippe Barre (INRA Lusignan, *Lolium* species). For each species, between five and ten seeds from self pollinated ears (when available or possible) were sown for further DNA extraction.

DNA extraction, PCR amplification and SSR detection

DNA was extracted from fresh leaves ground in liquid nitrogen using a CTAB protocol as previously described (Tixier et al. 1998). A set of 116 EST-SSRs (Zhang et al., 2005a) was selected (see electronically supplementary data) according to their ability to be transferable to the studied species. PCR reactions using the M13 protocol were carried out as described in Nicot et al. (2004) with an annealing temperature of 60°C for 30 cycles (30 sec 94°C, 30 sec 60°C, 30 sec 72°C) and 56°C for 8 cycles. Amplification products were visualized using an ABI PRISM®3100 Genetic Analyzer (Applied Biosystems). Finally, fragment sizes were calculated using GENESCAN and GENOTYPER softwares (Applied Biosystems), where different alleles are represented by different amplification sizes for tandem repeats. Two alleles are considered as identical when they show the same fragment size.

Estimation of PIC values and phylogenetic relationships

Allelic polymorphism information content (PIC) values were calculated in each case using the formula $PIC= 1-\sum (Pi)^2$, where P*i* is the proportion of the population carrying the i^{th} allele, calculated for each SSR locus (Botstein et al. 1980). As an estimate of the genetic diversity, the number of bands per locus and per individuals (NB) was computed as follows: NB = (N x 100)/(116 x Transferability (%) x Number of individuals) where N is the total number of bands observed for each species, 116 is the total number of EST-SSRs tested and Transferability is the percentage of EST-SSRs that give an amplification product on related species.

Results

Because EST-SSRs are derived from coding sequences, they frequently amplified several copies of the same gene in polyploid wheats resulting in the detection of multiple fragments. In this study, 53% of the 116 EST-SSRs amplified more than one band in *T. aestivum* and *T. durum* suggesting either the detection of homoeologous copies or duplication of the genes in

Table 1: Transferability of EST-SSRs from *T.aestivum* to related species. Transferability is computed as the percentage of EST-SSRs giving an amplification product on at least one of the accessions tested.

Species	**Sub-species**	**Genome**	**N of accessions**	**Transferability**
T. aestivum	*aestivum*	AABBDD	22	100%
	spelta	AABBDD	5	99.1%
	compactum	AABBDD	5	100%
	sphaerococcum	AABBDD	5	99.1%
	macha	AABBDD	5	99.1%
	vavilovi	AABBDD	5	99.1%
	petropavlovskyi	AABBDD	3	100%
T. turgidum	*carthlicum*	AABB	5	93.0%
	dicoccum	AABB	5	94.0%
	dicoccoides	AABB	5	94.7%
	durum	AABB	25	96.5%
	polonicum	AABB	5	87.7%
	turgidum	AABB	5	90.4%
T. monococcum	*monococcum*	AmAm	5	83.3%
	boeoticum	AbAb	5	83.3%
	urartu	AuAu	5	85.1%
Ae. speltoides		SS	4	79.0%
Ae. searsii		SsSs	2	74.6%
Ae. bicornis		SbSb	2	80.0%
Ae. longissima		SlSl	3	73.7%
Ae. tauschii		DD	5	76.3%
Ae. umbellulata		UU	2	77.2%
Ae. peregrina		UUSvSv	2	78.1%
Ae. comosa subven		MM	3	76.3%
Ae. ventricosa		DDMvMv	3	82.5%
Hordeum vulgare		HH	5	62.3%
Secale cereale		RR	5	72.8%
Lolium perenne			6	43.0%
Avena sativa			5	58.8%
Zea mays			5	40.4%
Oryza sativa			5	30.1%
Brachypodium			2	43.9%

the genome. Among these, 70% revealed only one band in the diploid species indicating that most of the fragments originated from homoeologous copies. When several bands were detected in diploid species, this could be due either to the presence of several copies of the genes or to a DNA mixture of heterogeneous varieties or to residual heterozygosity which can be frequent, especially in allogamous species. For these latter species, 69.3% (79) of the 116 EST-SSRs gave more than one band and among these, 32.5% (37) gave only one band in polyploid species. Only thorough sequence analyses of the different fragments and genetic mapping could allow concluding about the different hypothesis. Concerning the 47% of the EST-SSRs that amplified only one fragment in polyploid wheats, this suggests either that they were specific of one copy or that they amplified different copies but of the same size.

Characterization of the transferability of wheat EST-SSRs on a large set of species

We studied the transferability of these EST-SSRs to the different species. Transferability was considered as positive when an amplification product was detected on at least one of the different accessions used for each species. Considering this set of markers, transferability was complete (100%) for *T. aestivum* sub-species *compactum* and *petropavlovskyi*, and almost complete (99.1%) for sub-species *spelta*, *sphaerococcum*, *macha* and *vavilovi* (Table 1). Similarly, an average of 92.7% of transferability was observed for the six tetraploid subspecies. The highest value was observed for *T. durum* (96.5%) which was expected since this species is closely related to *T. aestivum*. Similar values were observed for *T. carthlicum*, *T. dicoccum* and *T. dicoccoides* (respectively 93%, 94% and 94.7%) suggesting also close relationships. Lower values were noticed for *T. turgidum* (90.4%) and *T. polonicum* (87.7%) indicating a larger divergence between these two species and bread wheat.

For the ancestral diploid species of hexaploid wheat, the transferability ranged from 76.3% for *Ae. tauschii* (DD) to 85.1.3% for *T. urartu* (A_uA_u), with an average of 83.9% (Table 1). A higher value was expected for *Ae. tauschii* since this species is known to be the donor of the D genome of hexaploid wheat, an event which occurred only recently (8,000 years ago). Thus, sequences should have diverged only weakly. For the species bearing the S genome which is supposed to be close to the B genome of bread wheat, transferability varied from 73.7% for *Ae. longissima* to 80% for *Ae. bicornis*. *Ae. speltoides* also exhibited a high value (79%) confirming that it can be considered as a good candidate for B genome donor

Table 2: Distribution of common bands between hexaploid wheat (*T. aestivum*) and other species. N is the number of different bands for each species. NB represents the number of bands per locus and per individual and is computed according to the number of accessions tested (see Table 1), the number of EST-SSRs tested (116) and their transferability to the species (see Table 1).

Species	NB*	N	*T. aestivum*aest	%CB§
*T. aestivum*aest	0.108	295	-	-
T. durum	0.093	237	177	74.7
*T. monococcum*mono	0.304	143	68	47.6
*Ae. speltoides*spelt	0.423	159	65	40.9
*Ae. tauschii*tauschi	0.269	122	70	57.4
*H. vulgare*ordeum	0.280	119	50	42.0
*S. cereale*ecale	0.292	140	54	38.6
*L. perenne*olium	0.388	155	39	25.2
*A. sativa*vena	0.256	100	61	61.0
*Z. mays*Maize	0.352	102	34	33.3
Rice	0.232	44	6	13.6

*NB: Number of bands/locus/individual = (N x 100)/(116 x TransferabilityTranferability (%) x Number of individuals)

§ percentage% of common bands with hexaploidbread wheat

species. For the A genome species, transferability was similar between the three sub species (*T. monococcum* 83.3%; *T. boeoticum*, 83.3%; *T. urartu* 85.1%) but was the highest for *T. urartu* indicating that this sub species was closer to *T. aestivum*. The transferability to other wild relatives of common wheat was also high ranging from 76.3% for *Ae. comosa* to 82.5% for *Ae. ventricosa*. This indicates that EST-SSRs can be very useful to study these species for which no genomic tools were previously developed.

We also analyzed the transferability to less related grass species but which are more or even largely studied (Table 1). It was found to be good with rye (*Secale cereale*, 72.8%), barley (*Hordeum vulgare*, 62.3%) and oat (*Avena sativa*, 58.8%). For rye-grass (*Lolium perenne*) and maize (*Zea mays*), transferability was respectively of 43% and 40.4%. For *Brachypodium*, about 44% of the EST-SSRs were transferable which confirmed that this species can be used as a model, especially for marker development (G. Moore, personal communication).

Globaly, we can conclude that transferability of EST-SSRs across a wide range of grass species was good indicating that they can be used either as genomic tools for orphan species or for comparative studies for cultivated crops.

Analysis of the allelic diversity within species

Polymorphism information content (PIC) values which provide an estimate of the discriminatory power of each EST-SSR locus were computed for *T. aestivum* and *T. durum* using respectively 60 and 52 EST-SSRs. For each species, EST-SSRs were chosen according to their ability of revealing polymorphism and to the absence of missing data. For *T. aestivum*, PIC values ranged from 0.08 to 0.80 (average 0.40 ± 0.20) which was similar to what was observed for *T. durum* (0.09 to 0.80; average 0.39 ± 0.19). For these two species, 355 bands were detected from the 116 amplified EST-SSRs (average 3.1 bands/locus). The primer pair CFE300 detected the largest number of bands (13) while the primer pair CFE264 detected the lowest number (2). We can conclude from this that EST-SSRs are polymorphic enough to serve as molecular markers in wheat.

The total number of bands was computed for 11 species, including *T. aestivum* and *T. durum*, the three ancestral diploid species of bread wheat and six members of the grass family (Table 2). For each of these species, we calculated the mean number of bands per locus and per accession (NB) which can be considered as representative of the genetic

diversity of the species. This value was corrected according to the transferability of the EST-SSRs for each species to take into account only markers giving amplification products (see M&Ms). *Ae. speltoides* showed the highest NB value (0.423) followed by *Lolium*, maize and *T. monococcum* species (respectively 0.388, 0.352 and 0.304) suggesting a high level of genetic diversity within these species. For the three formers, this can be explained by the fact that these are allogamous species. On the contrary, *T. aestivum* and *T. durum* exhibited the lowest values (0.108 and 0.093 respectively) indicating lower genetic diversity. As expected and except for maize, widely cultivated species (wheat and barley) exhibited lower genetic diversity compare to wild species such as *Ae. speltoides*, *T. monococcum* and *Ae. tauschii*. This confirmed that breeding contributed to the reduction of the diversity within cultivated species.

The percentages of common bands between species were also investigated (Table 2). *T. aestivum* and *T. durum* shared the highest percentage of common bands (74.6%) indicating a recent common ancestral origin. Among the three ancestral diploid species of bread wheat, *Ae. tauschii* had the highest percentage of common bands with *T. aestivum* (57.4%), followed by *T. monococcum* (47.6%) and *Ae. speltoides* (40.9%). This suggests that the A and D genome donors showed a better conservation with the corresponding genomes of common wheat while the B genome of wheat was less related to the B genome of *Ae. speltoides*. Among the other grass species, *T. aestivum* showed almost the same percentage of common bands with rye, oat and rye-grass (average 39%) while the percentages were higher for barley (43.5%) and lower for maize (33%) confirming that this latter species is less related to wheat. These results clearly suggest that the wild related species can serve as a source of new alleles for cultivated species such as wheat providing that they can easily be crossed with wheat and that recombination can occur between homologous chromosomes which is the case with *T. monococcum*, *Ae. speltoides* and *Ae. tauschii*.

Discussion

Bread wheat EST-SSRs were tested to evaluate their transferability on a larger set of grass species (18) compared to our previous work (eight; Zhang et al., 2005a) and to characterize their allelic diversity. We expected the transferability to be complete for all *T. aestivum* sub species while it was very high but not complete for *T. spelta*, *sphaerococcum*, *macha* and *vavilovi*. In each case, one SSR did not give any amplification product on the five accessions

tested. This was surprising since *T. spelta* and *sphaerococcum* are supposed to differ from *T. aestivum* by only recessive alleles at the respective loci *Q* (Miller, 1987) and *S1* (Rao, 1977). Those two genes are located on chromosome 5A (*Q*, Galiba et al., 1995) and 3D (*S1*, Rao, 1977). However, the two EST-SSRs that did not amplify on these species were located respectively on chromosomes 5B (CFE229) and 4A (CFE228) indicating that they were not involved (or associated to) in the expression of the speltoid spike and spherical kernel and that more than one gene explained the difference between *T. aestivum, T. spelta* and *T. sphaerococcum* sub species.

Our results confirmed that wheat EST-SSR markers are highly transferable across closely related genera (Eujayl et al. 2002; Gupta et al. 2003; Thiel et al. 2003; Yu et al. 2004a, b; Zhang et al. 2005a). These results contrast with those observed with genomic SSRs which are more genome specific and thus less transferable to related species (Röder et al. 1995; Sourdille et al. 2001; Kuleung et al. 2003). This is probably due to the fact that EST-SSRs originate from the coding regions which have a higher level of sequence conservation than intergenic regions.

On average, the number of alleles per locus computed on 22 hexaploid and 25 tetraploid wheats using our set of 116 EST-SSR markers was 3.1. This is lower than the average number observed using genomic SSRs (9.4, Balfourier, unpublished results). In our study, the mean of PIC values for EST-SSRs was 0.395 which is lower compared to the PIC values observed for genomic SSRs (Röder et al. 1995; Roussel et al. 2004). However, this is in agreement with other studies on EST-SSRs (Eujayl et al. 2002; Gupta et al. 2003; Nicot et al. 2004; Peng et al. 2005) and this is also higher than the values observed for RFLPs (0.30, Anderson et al. 1993). This suggests that EST-SSRs can constitute a good source of markers for genetic mapping. The lower level of the PIC values of the EST-SSRs compare to g-SSRs can be explained by the lower number of motifs present in EST-SSRs (in our set range 3-25, mean 6.15) while it was demonstrated that SSRs showing high number of repeats were more polymorphic (Guyomarc'h et al. 2002a; Thuillet et al. 2004). Accordingly, we should pay more attention to this point when considering polymorphisms of EST-SSRs in the future application.

Highest genetic diversity was observed in wild diploid species (*Ae. speltoides*, *T. monococcum*) but also in maize and oat while *T. aestivum* and *T. durum* exhibited the lowest values. This confirmed that the level of molecular polymorphism is low in these two latter species (Chao et al., 1989 ; Blanco et al. 1998). In addition, *T. aestivum* and *T.*

durum shared a high number of bands while the three ancestral diploid species of bread wheat (*T. monococcum*, *Ae. speltoides*, *Ae. tauschii*) had on average less than one half of common bands with these two species. In addition, allogamous species such as *Ae. speltoides*, rye-grass and maize showed highest diversity compare to autogamous species. Similar results were observed by Wang et al. (2005) who noticed that the level of polymorphism was significantly higher among species than within species and was related to the degree of out-crossing for each species. Polymorphism ranged from 57% for self-incompatible species to 20% for self-pollinated species. Our results suggest that the wild ancestral species may serve as sources of new alleles for cultivated wheats. This can be done by creating new synthetic wheats which can be used in breeding programs to develop new progenitors with enhanced capacities for stress tolerance useful in the view of a more sustainable agriculture. Previous studies of synthetic derived wheats have already revealed an increased diversity in the synthetics using AFLP (Lage et al, 2003) and SSR markers (Zhang et al, 2005b). It has also been proven that these synthetic wheats possess favorable qualitative (Kema et al, 1995; Ma et al. 1995; Lage et al, 2001; Mujeeb-Kazi et al. 2001) as well as quantitative traits (Villareal et al. 2001). However, they also carry a large number of unfavorable alleles and they thus need to be backcrossed to elite cultivars to produce agronomically acceptable progenitors.

As a conclusion, common wheat EST-SSRs showed a high level of transferability to numerous grass species including Pooideae species (oats, barley, wheats, rye) as well as Panicoideae species (maize). They can thus be used as a starting point for genomic studies of orphan wild wheat-related species in order to exploit these latter as reservoir of new alleles for wheat genetic improvement.

Acknowledgements

The authors thank gratefully G Gay and A Loussert for growing the plants and P Barre, P Barret and J David for providing with rye-grass, maize and *T. turgidum* sub species respectively. G Boutet and C Pont are also greatly acknowledged for their help in managing the robotics of the genotyping platform. This work was funded by the China Scholarship Council (CSC). All these experiments comply with the current laws of France.

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2.3Comments and perspectives

This work allows proposing some conclusions concerning the transferability of the wheat EST-SSRs and their exploitation for genomic studies in orphan species. First, this type of marker was proven highly transferable to wild wheat relatives. Second, these species are only poorly studied while we demonstrated that they might constitute a huge source of new variability for bread wheat improvement. It would be now interesting to try to introduce some new alleles at relevant genes such as biotic as well as abiotic resistance genes issued from these species within the elite wheat germplasm and look if this leads to an improvement of the introgressed lines. Introgressions would be easily followed and reduced using EST-SSRs.

3 Transferability of EST-SSRs between rice and wheat

3.1 Transferability from bread wheat to rice

In the first study (Zhang et al. 2005), the transferability of wheat EST-SSRs to rice was estimated to be 28.3%. However, when five accessions of rice are tested, the transferability is increased and reached 30.1% of wheat EST-SSRs that are transferable to at least one rice accession. Among these latter, 60% gave an amplification product on at least two rice accessions. In order to investigate the syntenic relationships between wheat and rice chromosomes, the EST-SSRs were first assigned to wheat chromosomes and we surveyed their location on rice chromosome by blasting wheat EST bearing SSRs against the 12 rice pseudo-molecules. Our results were consistent with the known structural relationships between wheat and rice genomes as revealed by previous studies (Devos and Gale. 1997; Sorrells et al. 2003). For example, wheat ESTs bearing SSRs matching sequences on rice chromosome 1 (R1) were largely from wheat homoeologous group 3, whereas R2 and R3 were generally related to wheat homoeologous groups 6 and 4, respectively.

As a conclusion, wheat EST-SSRs show quite a high level of transferability to rice. Wheat ESTs containing SSRs also demonstrated the structural relationships between wheat and rice genomes. Therefore, SSRs derived from bread wheat can be used in comparative genomics involving rice and wheat.

3.2 Transferability from rice to bread wheat

The recent sequencing of the rice (*Oryza sativa*) genome (IRGSP, 2005; http://rgp.dna.affrc.go.jp/IRGSP/) has provided thousands of potentially new markers in order to increase the grass map densities. However, not all the markers can be easily transferred from rice to wheat and only genes are well conserved between the two species. The markers should thus be derived from low-copy sequences, which are unfortunately less polymorphic than the others. This is why rice EST-SSRs can be of main interest to saturate wheat genetic maps. At the UMR INRA-UBP, we recently decided to focus our efforts on the establishment of the physical map of chromosome 3B (Safar et al. 2004, Feuillet et al. unpublished results). This chromosome bears numerous genes involved in a lot of important traits such as grain yield and seed weight (Berke et al. 1992a,b), kernel color (Sears 1944; Metzger and Silbaugh 1970; Nelson et al. 1995), chromosome pairing (Sears 1982; Dong et al. 2002), seed dormancy (Osa et al. 2003), numerous resistance genes (Mcintosh et al. 1977; Hare and Mcintosh 1979; Ma and Hughes 1995; Liu and Anderson et al, 2003) and isozymes (Hart et al. 1993; Mcintosh et al. 1998). In order to

anchor accurately the physical map to the genetic map, we thus need to develop more molecular markers. Despite the fact that it is the largest among the wheat chromosomes (Dvorak et al. 1984; Gill et al. 1991), comparisons with chromosomes related to wheat group 3 from other species such as chromosome 1 from rice indicated that this group is the best conserved in gene content and order (Gale and Devos 1998; Sorrells et al. 2003; La Rota and Sorrells 2004).

Therefore, the purposes of this part were (a) to investigate the characteristics and the diversity of the SSRs issued from genes located on rice chromosome 1; (b) to develop rice EST-SSR markers from the distal region of the long arm of rice chromosome 1 and to study their transferability to wheat and its close and wild relatives; (c) to assign amplifying primer pairs to chromosomes by using nulli-tetrasomic (NT) lines to confirm the syntenic relationships with wheat homoeologous group 3 and to investigate their potential for comparative mapping.

3.3 Analysis of rice EST-SSRs

3.3.1 Characteristics of SSRs in genes on rice chromosome 1

In the study of rice EST-SSRs, 12,078 perfect and imperfect SSRs were identified from 4,100 genes containing SSRs on rice chromosome 1. Like in wheat ESTs, the trinucleotide repeats were the most common (60%) in rice chromosome 1 genes in CDS as well as in introns, (CCG)n motif being the most abundant. Concerning the other types of motifs, AC dinucleotide was the most frequent in whole genes and in introns, while CG was more abundant in CDS. In addition, four-repeat motifs occurred as frequently in the whole genes, CDS and introns.

3.3.2 Amplification of rice EST-SSRs on rice

Among the 4,100 genes containing SSRs, 200 were randomly selected in two well conserved regions between rice chromosome 1 and homoeologous group 3 of wheat. A total of 332 SSRs were found among which 170 were located in CDS and 162 in introns. From the CDS sequences with SSRs, primers were designed for 106 EST-SSRs among which 102 gave an amplification product on rice (96%).

On rice, some differences were observed between the expected and the observed size of the amplification product. Among the 102 primer pairs, four (3.9 %) gave an amplification product smaller than expected, suggesting (1) the occurrence of deletions within the

Table 3-3: Chromosomal assignment on wheat NT lines for 18 EST-SSRs derived from rice chromosome 1

Marker	Motif	Repeat	Forward primer	Reserve primer	Chromosome
cfr5	cctccg	4.2	GGTCGGGATGTACCAGCA	TGAACGCCTCGAACAGCC	1B, 6B
cfr17	cag + cag	4+4	TCGCAGTCAGTCGGGAGC	GCCTGGCTTGTAGCTGTAATC	3D
cfr20	gcc	7	GACAGGAAGGTGTCGTGC	GGATGGTTATCGACTCCG	3B, 3D
cfr22	cggcga	4	GCGGTGCTCTGGGTGAGG	GACGGCGTCTCCTCGATC	1B, 3A
cfr35	cgc	6	CTCGCCGTGTTCCTCCAC	CGCCCTGTACGTCTCGCT	7A, 7B
cfr37	gcg	4	CGGAGCAGATGAGGGAGATC	CACCGCTCGACGACGAG	1D
cfr39	ggc	4	CCACAAGCTCCTCCCGTT	CGAACACGCACTGGAAGTAC	3A, 3B
cfr46	gtc	4	CCTCATCGACGCCACGTAC	CGAAGACGCACGACTTGC	3B, 3D
cfr52	gtc	4	ATGGACGTGGAGAAGGTGG	CAGAAGAAAGACAGGCAGGG	2AL, 6B, 7B
cfr60	gaa+agc	4+5	GGATCAGGCTCCTCCTCG	CAGCCGCAGCTCCTTCAT	3A, 3B
cfr68	cca	4	GAGTCCCTCCCCTTCTCCCT	CGAATGCGAATGCGGATG	3D
cfr75	tga	4	GGTGCTTTCGATGGTGTTC	ATCCCAAGGCTCCATAAG	5B, 7A
cfr79	cga	4	CGACCAGGAAATGTGGCG	CGATGTCCACGAGGCTCC	3A, 3B
cfr88	aga	3	GCAGGTAGAGGAGAGGATGATG	ATGAGCGGCTCCGACATG	1D, 4A, 4B
cfr93	acg	4	GGAGCAGCGGATGAACAG	GGACTCCATCAGCCACCG	1A
cfr97	gccgac	4	CCCACCAACGACTTCAAC	AAGGAGCAGACGAAGTGC	1A, 1B
cfr11	gcg	5	GAGCCAAAGCCAAAGCCC	GCTACCGCACCCAGTCCT	not assigned
cfr36	acg	5	CTCTCGGTGACCCGCTGT	GAGCCACGCCAAGCACTC	too complex

genomic sequences; (2) a lack of specificity of some primer pairs which may have amplified a different copy belonging to the same multigenic family; (3) a slight variation between the amplified copy and the consensus sequence. Only one primer pair (CFR35) yielded two products: one band was consistent with expected product length while the other was larger, suggesting the possible simultaneous amplification of a different copy of the same gene during the PCR.

3.3.3 Transferability of rice EST-SSRs

The transferability was investigated from rice to *T. aestivum* (eight common wheat varieties), *T. durum*, *T. monococcum*, *Ae. speltoides*, *Ae. tauschii*, *Secale cereale*, *Hordeum vulgare*, and *Agropyrum elongatum*. Concerning the eight common wheat lines, 45 (44%) out of 102 primer pairs gave one product in at least one cultivar and 29 (28.4%) amplified in at least two cultivars. Eighteen (17.6%) amplified in all eight cultivars among which, 15 were trinucleotide repeats, while the remaining three were hexanucleotide repeats. For transferability from rice to other species, similar results were observed for *T. durum* (AABB), *T. monococcum* (AA) and *Ae. speltoides* (BB) where transferability on most of the cultivars tested account for about 18%. For the other species, 13.7% and 14.7% of rice EST-SSRs were transferable to *Ae. tauschii* (DD) and rye (RR) respectively while lower levels of transferability were found for barley (8.8%) and *Agroprum elongatum* (6.9%).

3.3.4 Assignment to wheat chromosomes

The 18 primer pairs that yielded products in almost all common wheat cultivars were used to test on Chinese Spring aneuploid lines. Overall, 16 markers were assigned to wheat chromosomes. The two remainings were not assigned because: one gave too complex profiles to be properly assigned; another amplified a product in all NT and DT lines suggesting different products of the same size on at least two of the three homoeologous chromosomes. Among the 16 markers, four were assigned to a single locus while the 12 others were assigned to a maximum of three loci. Most of them (8) were found at loci located on the same homoeologous group (Table 3-3). The 29 EST-SSR loci were assigned to wheat chromosomes, 10, 14 and 5 loci being placed on the A, B and D genomes respectively. No locus was found on chromosomes 2B, 2D, 4D, 5A, 5D, 6A, 6D and 7D. The distribution on other chromosomes ranged from 1 locus on chromosome 2A, 4A, 4B, and 5B to 5 loci on chromosome 3B. According to the syntenic relationships between

Table 3-4: Distribution of rice EST-SSR loci according to their assignment to wheat chromosomes and homoeologous groups

Homoeologue group	**1**	**2**	**3**	**4**	**5**	**6**	**7**	Total
chromosome **A**	2	1	4	1	0	0	2	10
chromosome **B**	3	0	5	1	1	2	2	14
chromosome **D**	1	0	4	0	0	0	0	5
Total	6	1	13	2	1	2	4	29

wheat homoeologuous group 3 and rice chromosome 1, the marker derived from rice chromosome 1 should have been assigned to group 3 chromosomes of wheat. In our study, 8 out of 16 assigned rice markers were located on wheat chromosomes 3A, 3B, and 3D and overall, 13 loci were found on wheat homoeologuous group 3 chromosomes with no significant bias observed, 4, 5 and 4 loci being located on wheat chromosomes 3A, 3B, and 3D respectively (Table 3-4). In addition, six loci were assigned to wheat homoeologous group 1 where a well known duplication exists with homoeologous group 3 (Salse J. personal communication)

As a conclusion, the SSRs derived from coding DNA sequence on rice chromosome 1 also showed quite a high level of transferability across its distant relative (Triticeae) and thus may be successfully used for comparative genomics studies such as genome analysis, localization of expressed genes, survey of orthologous relationship, and fine mapping of regions of interest.

3.4 Discussion, comments and perspectives

In this part, 96.2% of the primer pairs successfully amplified products in rice species, and about 80% of them produced strong and clear profiles. About 96% of the primer pairs yielded fragments of the expected size contrary to genomic SSRs, where only 36% did in common wheat, with many of them resulting in a smear (Röder et al. 1995). For 18 primer pairs that amplified in almost of the lines tested, 16.7% (3/18) exhibited polymorphism among the eight wheat cultivars. This was much lower than the results described in previous studies (about 25%, Eujayl et al. 2001; Gupta et al. 2003; Thiel et al. 2003; Gao et al. 2004; Nicot et al. 2004), and than that observed for genomic SSRs (53%, Eujayl et al. 2001). Two reasons can explain this fact: 1) these rice primer pairs that amplify in common wheat, most likely reside in well conserved regions between two species; 2) the characteristics of EST-SSRs, *i. e.* a lower number of repeat units, can also account for this lower percentage of polymorphism. Three polymorphic primers were screened in two mapping populations (ITMI and Courtot X Chinese Spring segregating populations), for genetic mapping in common wheat. But they failed to be mapped because very complex profiles were yielded.

SSRs derived from ESTs are especially valuable as molecular markers because they are derived from gene transcript and are more likely to be conserved among species. Therefore these markers may be more transferable than genomic SSRs (Gao et al. 2003; Yu et al.

2004b; Gupta et al. 2003; Zhang et al. 2005). However, because rice is very distant relative to wheat, a lower level of transferability noticed on at least one line was observed for rice EST-SSRs toward wheat and its close and wild relatives: *T. aestivum* (44%), *T. durum* (18.6%), *T. monococcum* (17.6%), *Ae. speltoides* (17.6%), *T. tauschii* (13.7%), rye (14.7%), barley (8.8%) and *Agroprum elongatum* (6.9%). The similar transferability from common wheat to rice was reported in previous study (45%, Yu et al. 2004a). Considering those amplifying in at least two lines, the transferable rate was 28.4% from common wheat to rice which was consistent with the transferability from wheat to rice in our previous study (28.3%, Zhang et al. 2005). When primers amplifying in all cultivars were investigated, only 17.6% (18) of transferability was detected. This suggested that DNA sequence was well conserved for 18 primers through 65 million years' divergence, while several bases in the flanking regions were subjected to mutate for the other primers.

In order to evaluate the utility of EST-SSRs as anchor markers we assigned these 18 markers to wheat chromosomes by using Chinese Spring NT and DT lines. Sixteen markers were assigned to wheat chromosomes. Because these markers were developed from rice chromosome 1, as expected, most of them (50%) were located on wheat homoeologous group 3, according the orthologous relationship between rice and wheat while the others were on the other groups. Similar results are mentioned by Munkvold et al (2004) who found that 59% of wheat group 3 mapped–EST unigenes showed homology to rice 1 when constructing group 3 chromosome bin map of wheat. Our results supported the structural relationships between wheat and rice reported in previous comparative maps at the macro level. In our study, 12 among the 16 markers were assigned to a maximum of three loci. Sixty seven percent of them were found at loci located on the same homoeologous group. Likewise, 70% of markers detected multiple loci in the study for assessing the efficiency of EST-SSRs in comparative mapping (Yu et al. 2004b). For instance, CFR22 amplified two fragments but only one was assigned to wheat chromosome 3A while the other was located on wheat chromosome 1B. This can be explained by the fact that these markers developed from expressed genes were frequently duplicated in the genome. Moreover, most of the additional loci mapped to wheat homoeologous group 1. A duplication between wheat homoeologous groups 3 and 1 was already evidenced (Salse J personal communication). Our results are thus consistent with what was previously described.

Even in the most conserved region, all wheat chromosome deletion bins containing only

sequences from one rice chromosome were rare (Sorrells et al. 2003). Most blocks of conserved order span regions of rice chromosome 1 matched to wheat ESTs mapped to other homoeologous groups (Munkvold et al. 2004). The similar case was observed in our study; half of 16 markers were assigned elsewhere in the wheat genome, such as wheat group 1, 4 and 7 etc. As reported by Liu and Anderson (2003), among 68 STS markers developed from rice chromosome 1S in order to target a wheat QTL for *Fusarium* head blight resistance, 28 were assigned to chromosome 3BS. Likewise, one third of the ESTs from wheat group 3S identified orthologs on rice chromosome 1 by *in silico* analysis (Francki et al. 2004). These results suggest that there has been an abundance of rearrangements, insertions, deletions, and duplications during cereal evolution that will complicate the utilization of many regions of the rice genome for cross-species transfer of information. It is also expected that this disruption in the colinearity of genes will greatly complicate map-based cloning and selection of linked markers.

In summary, it is concluded that the SSRs derived from the functional portion of the genome of rice showed a good level of transferability across its distant relatives (Triticeae) and thus may be successfully used for comparative genomics studies such as genome analysis, localization of expressed genes and survey of orthologous relationship.

The investigation for reciprocal transferability of EST-SSRs between wheat and rice showed very similar results with 28.3 % of transferability from wheat to rice and 28.4% from rice to wheat. This means that quite a high proportion of expressed sequences, even those bearing SSRs have remained well conserved after the divergence between the Bambusoideae and the Pooideae during several million years. Amplification products yielded by EST-SSRs assigned on the wheat homoeologous groups 3 chromosomes should be sequenced in order to obtain more information to explore the evolution mechanisms of SSRs in orthologous region in grass genomes.

4 Phylogenetic studies of Triticeae using wheat EST-SSRs

Since wheat EST-SSRs are transferable to a large number of wheat related species. It was expected that they could be used for studying the relationships within the Triticeae species. This part was the subject of the third manuscript which was accepted for publication in Theoretical and Applied Genetics. This paper discusses the relationships among the polyploid wheat species and also the relations between ancestral diploid species and polyploid species.

4.1 Transferable bread wheat EST-SSRs can be useful for phylogenetic studies among the Triticeae species (Theor Appl Genet, DOI 10.1007/s00122-006-0304-4)

Theor Appl Genet
DOI 10.1007/s00122-006-0304-4

ORIGINAL PAPER

Transferable bread wheat EST-SSRs can be useful for phylogenetic studies among the Triticeae species

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Received: 8 February 2006 / Accepted: 28 April 2006

Abstract The genetic similarity between 150 accessions, representing 14 diploid and polyploid species of the Triticeae tribe, was investigated following the UPGMA clustering method. Seventy-three common wheat EST-derived SSR markers (EST-SSRs) that were demonstrated to be transferable across several wheat-related species were used. When diploid species only are concerned, all the accessions bearing the same genome were clustered together without ambiguity while the separation between the different sub-species of tetraploid as well as hexaploid wheats was less clear. Dendrograms reconstructed based on data of 16 EST-SSRs mapped on the A genome confirmed that *Triticum aestivum* and *Triticum durum* had closer relationships with *Triticum urartu* than with *Triticum monococcum* and *Triticum boeoticum*, supporting the evidence that *T. urartu* is the A-genome ancestor of polyploid wheats. Similarly, another tree reconstructed based on data of ten EST-SSRs mapped on the B genome showed that *Aegilops speltoides* had the closest relationship with *T. aestivum* and *T. durum*, suggesting that it was the main contributor of the B genome of polyploid wheats. All these results were expected and demonstrate thus that EST-SSR markers are powerful enough for phylogenetic analysis among the Triticeae tribe.

Communicated by M. Sorrells

Electronic Supplementary Material Supplementary material is available to authorised users in the online version of this article at http://dx.doi.org/10.1007/s00122-006-0304-4.

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Introduction

The grass family (Poaceae or Gramineae) comprises the most important cultivated crops in the world. It includes all the cereals as well as forage crops, sugar cane, or bamboos. There are more than 10,000 grass species distributed among 651 genera which originated and diverged during about 60 million years. They are grouped in five sub-families among which the Bambusoideae, the Panicoideae, and the Pooideae which include the cultivated species such as wheat, barley, rice, and maize. Wheat species and their close relatives belong to the Pooideae sub-family and to the Triticeae tribe which comprises the genera *Triticum* (wheat), *Aegilops*, *Secale* (rye), and *Hordeum* (barley). Within this latter tribe, diploid as well as polyploid species can be found. In the Triticeae, the basic chromosome number is seven and the genomes are designated by letters. These genomes are not perfectly identical but show high levels of similarity and are thus named "homoeologous." Wheat polyploid species originated from hybridizations between either diploid species or diploid and polyploid species followed by natural genome doubling. For example, the hexaploid bread wheat (*Triticum aestivum* L.em.Thell. $2n = 6x = 42$) is an allopolyploid species (AABBDD) originated by hybridization of a AA-genome diploid species related to *Triticum urartu* ($2n = 2x = 14$, Dvorak et al. 1993; Huang et al. 2002) with a BB-genome diploid species from the *Sitopsis* section giving rise to *Triticum dic-*

occoides species (AABB), followed by an additional hybridization with a DD-genome diploid species, *Aegilops tauschii* ($2n = 2x = 14$, McFadden and Sears 1946). The origin of the B genome remains controversial but *Aegilops speltoides* seems to be the most likely living relative of an extinct or yet to be discovered B-genome donor species (Sarkar and Stebbins 1956; Riley et al. 1958; Rees and Walters 1965; Natarajan and Sharma 1974; Chen et al. 1975; Jaaska 1980; Hassan and Gustafson 1996; Maestra and Naranjo 1998).

Grasses taxonomy and phylogeny were initially based on morphological and physiological traits. However, discrimination between species was sometimes limited because of a lack of relevant descriptors, or divergence was overestimated because of the difficulty in scoring some characters. Morphological traits have thus been supplanted first by isozymes which have provided valuable insights into the phylogeny among the genera and species (McIntyre 1988) and later by DNA-based molecular markers. The results obtained with these tools were relatively consistent with the general taxonomic information provided by earlier morphological, physiological, and isozyme markers (Monte et al. 1993). Especially, SSRs were extensively explored in wheat evolutionary studies because of their high polymorphism level, their co-dominant inheritance, and their reproducibility (Plaschke et al. 1995; Donini et al. 2000; Prasad et al. 2000; Manifesto et al. 2001; Ben Amer et al. 2001; Leisova and Ovesna 2001; Röder et al. 2002; Zhang et al. 2002; Roussel et al. 2005). However, interspecific phylogenetic studies in wheat using SSRs were limited to exploration of the relationships between *Ae. tauschii* and the D genome of bread wheat (Lelley et al. 2000) because of the limited transferability of genomic SSRs to related species (Sourdille et al. 2001).

Recently, Zhang et al. (2005) demonstrated that bread wheat EST-derived SSR markers (EST-SSRs) developed from EST sequences showed a high level of transferability to close and wild relatives of wheat because they are derived from conserved coding regions. In addition, they are still a source of information for assessing genetic relationships (Eujayl et al. 2001, 2002) as suggested by Bandopadhyay et al. (2004), who studied DNA polymorphism among 18 species of *Triticum–Aegilops* complex and were able to construct a dendrogram separating the diploid and tetraploid species.

In this paper, we studied the genetic similarity among 150 accessions from 14 different Triticeae species representing 16 different genomes using a set of 73 EST-SSRs that we recently developed (Zhang et al. 2005) with the final aim as the evaluation of the potential of wheat EST-SSRs for phylogenetic studies among different diploid, tetraploid, and hexaploid sub-species of the Triticeae tribe.

Materials and methods

Plant material

A total of 150 accessions representing 14 species and 16 genomes of the Triticeae tribe were used (see Supplementary Table 1, seeds available on request). This included diploid and polyploid species and autogamous as well as allogamous species. Similar numbers of accessions (between two and five) for each species were randomly chosen among our collection, except for *T. aestivum* and *T. durum*, where 23 varieties were selected for each species. Seeds were obtained from the Centre of Biological Resources on Cereal Crops (INRA-Clermont-Ferrand, France) and from Jacques David (INRA Montpellier, France, tetraploid and *Aegilops* species). For each species, between five and ten seeds from self-pollinated ears (when available or possible) were sown for further DNA extraction.

DNA extraction, PCR amplification and SSR detection

DNA was extracted from fresh leaves ground in liquid nitrogen using a CTAB protocol as previously described (Tixier et al. 1998). A set of 73 EST-SSRs (Zhang et al. 2005; http://www.wheat.pw.usda.gov/ITMI/EST-SSR/) was selected (Supplementary Table 2) according to their ability to be transferable to the studied species. PCR reactions using the M13 protocol were carried out as described in Nicot et al. (2004) with an annealing temperature of 60°C for 30 cycles (30 s 94°C, 30 s 60°C, 30 s 72°C) and 56°C for 8 cycles. Amplification products were visualized using an ABI PRISM®3100 Genetic Analyzer (Applied Biosystems). Finally, fragment sizes were calculated using GENESCAN and GENOTYPER softwares (Applied Biosystems), where different alleles are represented by different amplification sizes for tandem repeats. Two alleles are considered as identical when they show the same fragment size. To discriminate between PCR failure and null allele, PCR reactions were done twice.

Estimation of genetic similarity

Because only a limited number of accessions was tested for each species (between two and five), it was not possible to accurately measure the allelic frequency for each SSR and to use statistical analyses such as AMOVA or *F* statistics. For phylogenetic studies, we

decided thus to generate a binary matrix as followed: presence of an amplified product of a given size was scored as "1" while the absence of the same amplification product was scored as "0." The binary data were used to compute the distance matrix as 1—the Jaccard's similarity coefficient (Jaccard 1908). Phylip software was used to identify the genetic similarity (Felsenstein 1993). As all the species studied belong to the Triticeae tribe, the assumption of a molecular clock was acceptable. Therefore, the dendrograms were obtained by the UPGMA clustering method. The reliability and goodness of fit of dendrograms obtained from EST-SSRs data were tested through bootstrapping based on 100 samples (Felsenstein 1985). This led to 100 dendrograms summarized in a consensus tree which indicated the proportion of bootstrapped trees showing that same clade.

Results

Transferability of the EST-SSRs to the different species was high and ranged from 100% for *T. aestivum* sub-species *compactum* and *petropavlovsky*, to 62.3% for barley (Zhang LY et al., unpublished results). Since transferability was not complete, null alleles were not considered and were quoted as missing data since there was a higher probability that the lack of amplification was due to the presence of numerous mutations in the flanking sequences, which are obviously different among the species rather than to a deletion of the genes which could have been considered as similar events. This also justifies the choice of the Jaccard distance index (Jaccard 1908) which does not consider as informative a shared absence of a given trait (here an amplification product). However, we only retained markers for which the percentages of missing data were low and never exceeded the maximum of one missing data/locus for each species. Thus, estimation of genetic distances was quite accurate.

Clustering of the diploid grass species

We selected the 46 accessions of diploid species (Supplementary Table 1), including the A-, B- (or S), and D-genome donors of hexaploid wheat, *Aegilops umbellulata* (UU), *Aegilops comosa* (MM), barley (HH), and rye (RR). The clustering of the lines was based on 1,170 informative fragments produced from 73 EST-SSRs. The Jaccard genetic distance coefficients (Jaccard 1908) ranged from 0.307 between *Triticum monococcum* accessions 68191 and 68212 to 0.978 between *Aegilops tauschii* accession 15 and rye accession SCW 3. The consensus tree obtained (Fig. 1) showed that all the accessions bearing the same genome were clustered together without ambiguity, the bootstrapped values for each group being generally higher than 85%. The A-genome sub-species were divided into two groups, one including *T. urartu* sub-species only, the other where *T. monococcum* and *T. boeoticum* sub-species were clustered together. This latter group presented two clusters, one mainly corresponding to *T. monococcum* sub-species, the other one to *T. boeoticum* sub-species. Within the A-genome sub-species, the *T. boeoticum* accessions 68182 and 68184 and the *T. urartu* accession 78096 were not clustered with the other accessions of the same sub-species. *T. boeoticum* accession 68182 was associated with *T. monococcum* sub-species while *T. boeoticum* accession 68184 was with *T. urartu* sub-species. On the contrary, the *T. urartu* accession 78096 was clustered with *T. boeoticum* sub-species. However, the bootstrap values of the nodes leading to these groups were, respectively, 23 and 26% indicating that these groups were not highly reliable.

Similarly, the B-genome sub-species were divided into two groups, one where *Aegilops searsii*, *Aegilops bicornis*, and *Aegilops longissima* were clustered together, while *Ae. speltoides* was apart. Within the B-genome sub-species, the *Ae. bicornis* accession 471323 (AEBIC-47) was associated with two of the *Ae. longissima* accessions while *Ae. longissima* accession 330486 (AELON-330) was associated with the *Ae. bicornis* accession 70 (AEBIC-70). Concerning the relationships among the three diploid ancestors of hexaploid wheat, the *Ae. tauschii* species was more closely related to the B-genome species than to the A-genome species. However, this grouping was found in less than 60% of the trees indicating that relative position of these species was not accurate. *Ae. umbellulata* (UU) and *Ae. comosa* (MM) were clustered together and formed a group which was associated with the one including B- and D-genome sub-species in 87% of the dendrograms. Barley was found to be closer to all these wild species compared to rye but in less than 60% of the trees. This indicated that the relative positions of barley, rye, and wheat related diploid wild species need to be clarified.

From this tree, we can conclude that wheat EST-SSRs are powerful enough to assess the genetic variability of wheat diploid relatives. Also, clustering of some accessions should be reconsidered according to the results we report.

Clustering of the tetraploid grass species

Similarly, we used the 48 accessions of tetraploid grass species (Supplementary Table 1) to construct a

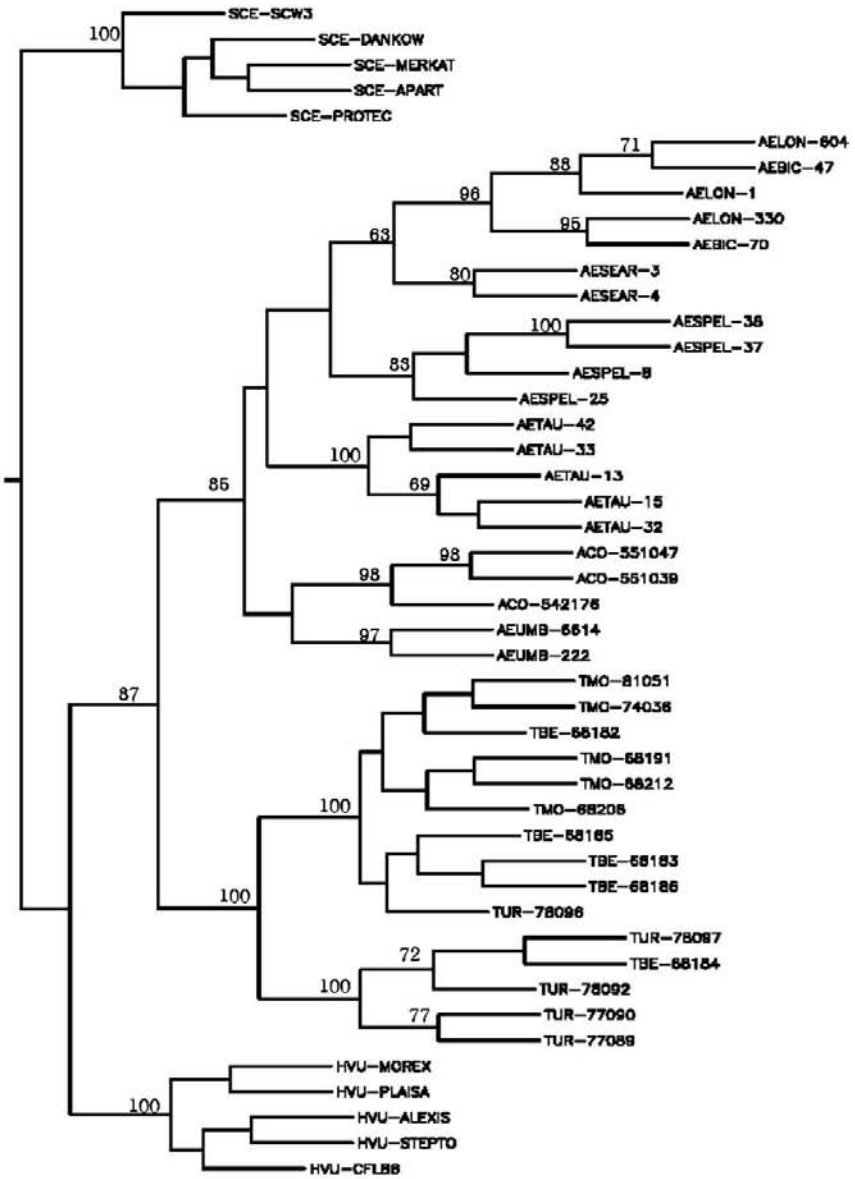

Fig. 1 Consensus tree of 46 accessions of diploid species reconstructed from 100 UPGMA trees obtained from data resampled in a set of 73 EST-SSRs. The accession codes are the same as those indicated in Supplementary Table 1 (SCE rye, AELON *Ae. longissima*, AEBIC *Ae. bicornis*, AESEAR *Ae. searsii*, AESPEL *Ae. speltoides*, AETAU *Ae. tauschii*, ACO *Ae. comosa*, AEUMB *Ae. umbellulata*, TMO *T. monococcum*, TBE *T. boeoticum*, TUR *T. urartu*, HVU barley). The branch lengths are proportional to the number of times that each group appeared. Additionally, *numbers* indicated bootstrap values larger than 60%

dendrogram. The clustering of the lines was elaborated based on 167 informative fragments produced from 73 EST-SSRs. The Jaccard genetic distance coefficients (Jaccard 1908) ranged from 0.322 between *Triticum turgidum* accessions 7786 and 387456 to 0.674 between *Triticum carthlicum* accession 94753 and *T. dicoccoides* accession 467014. The consensus dendrogram obtained from bootstrapped data is presented in Fig. 2. Separation between the different sub-species was less clear compared to the diploid species. However, all the

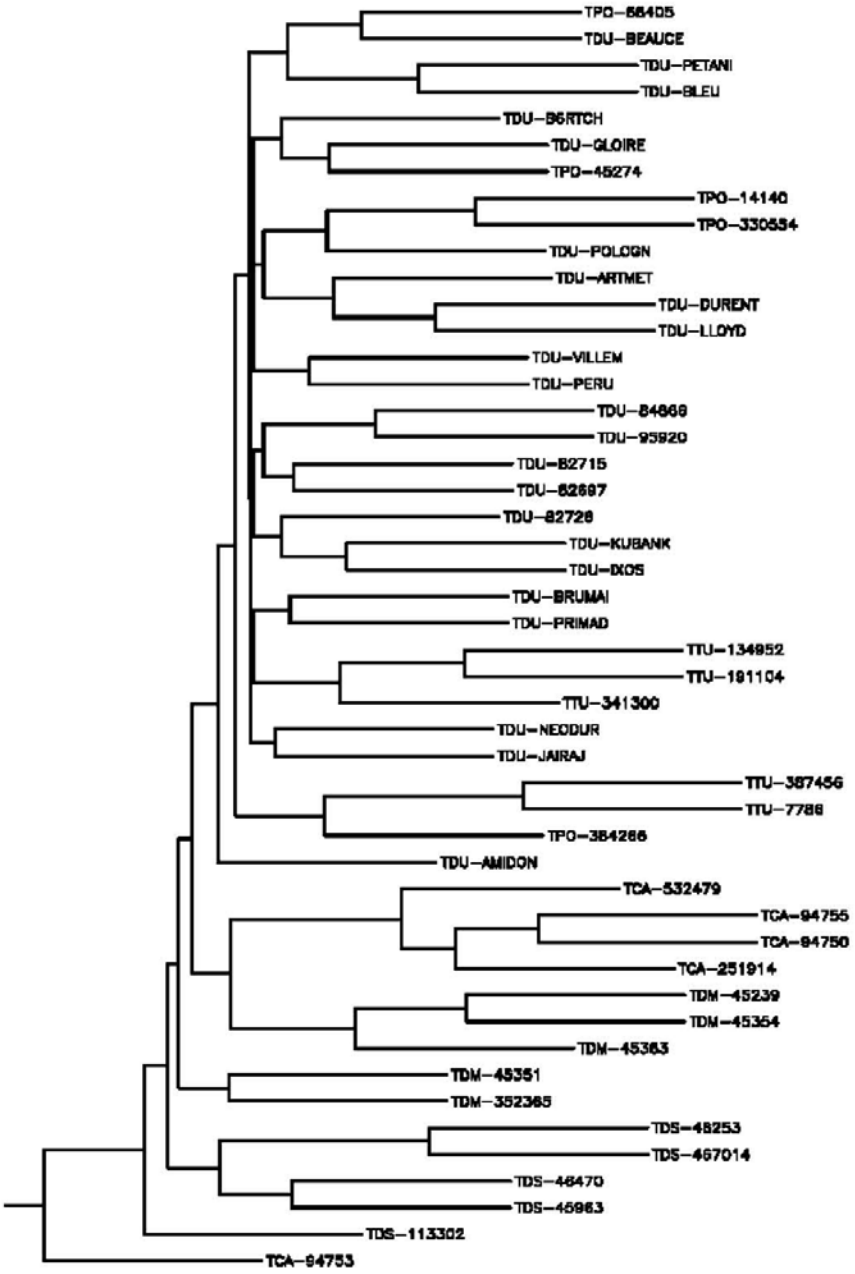

Fig. 2 Consensus tree of 48 accessions of tetraploid species reconstructed from 100 UPGMA trees obtained from data resampled in a set of 73 EST-SSRs. The accession codes are the same as those indicated in Supplementary Table 1 (TPO *T. polonicum*, TDU *T. durum*, TTU *T. turgidum*, TCA *T. carthlicum*, TDM *T. dicoccum*, TDS *T. dicoccoides*). The branch lengths are proportional to the number of times that each group appeared. Additionally, *numbers* indicated bootstrap values larger than 60%

T. durum accessions were grouped except the French variety "Amidonnier Blanc Barbu." Most of the *polonicum* (4/5) and the *turgidum* (3/5) sub-species were associated with the same group. The *carthlicum* subspecies were associated with the *dicoccum* sub-species while the *dicoccoides* sub-species formed a separate

cluster. The *carthlicum* accession 94753 was completely isolated.

From this tree, we can conclude that the relationships among all the wheat tetraploid species are closer compared to what was observed between the diploid species. This suggests that tetraploid species are more closely related than the diploid species indicating that they diverged more recently.

Clustering of the hexaploid grass species

Fifty accessions of hexaploid wheat species (Supplementary Table 1) were used to construct a dendrogram from 207 informative fragments produced from 73 EST-SSRs. The Jaccard genetic distance coefficients (Jaccard 1908) ranged from 0.231 between *Triticum aestivum* ssp. *macha* Landrace 4 and *T. ae.* ssp. *macha* accession 102V to 0.646 between *T. aestivum* cv Aurore and *T. ae.* ssp. *compactum* accession Lo To Mai. The consensus dendrogram obtained from bootstrapped data is presented in Fig. 3. Two groups were obtained. The first one was obtained in 48% of the bootstrapped trees and was made of most of the *T. aestivum* cultivars (15/22). On the contrary, the second one included all the others plus seven *T. aestivum* cultivars. Within this group, only *spelta* and *macha* sub-species were clustered together but UPGMA trees were found in less than 60% of the cases indicating that the clustering was not sure. The European accessions trended to cluster while the Asian ones were closer to each other.

Our results suggest that *T. aestivum* varieties seem to be split into two main groups: one includes most of the European and Asian (Korea, China, Afghanistan, Pakistan) varieties which is closer to other *T. aestivum* sub-species (*macha*, *spelta*...), the other includes the remaining varieties from other countries. This may indicate a common ancestral origin for each of the two groups.

Relationships between the A and B genomes for bread wheat and related diploid species bearing homoeologous genomes

Two sets of 16 and 10 markers evenly located on the A and B genomes of hexaploid wheat, respectively (Zhang et al. 2005), were selected to investigate the relationships among these two genomes of hexaploid, tetraploid, and diploid wheat species.

For the A genome, 244 informative fragments obtained from 16 EST-SSRs assigned to the A genome of bread wheat were used. The Jaccard genetic distance coefficients (Jaccard 1908) were calculated using these data and ranged from 0.052 between *T. durum* cv Ixos and Kubanka to 0.973 between *T. monococcum* accession 74036 and *T. aestivum* cv Seuseun 27. The consensus tree obtained from bootstrapped data is shown in Fig. 4. Tetraploid species (AB) were clustered together and were closely related to hexaploid species (ABD). Surprisingly, the French varieties Apache and Ornicar were closer to *durum* species than to *aestivum* species. Within the hexaploid species, the same separation into two groups as previously described (see previous section) was observed but the bootstrap values were not highly significant. On the contrary, the diploid species bearing the A genome were divided into two groups, one including all the accessions of *T. urartu*, the other grouping the sub-species *T. monococcum* and *T. boeoticum*. Within this latter group, *monococcum* and *boeoticum* sub-species were separated except *T. monococcum* accession 68191 which was linked to the *boeoticum* sub-group. *T. urartu* showed a closer relationship with *T. aestivum* and *T. durum* compared to *T. monococcum* and *T. boeoticum* confirming that *T. urartu* is the most probable ancestor of the A genome of polyploid wheats.

Similarly, 117 informative fragments obtained from 10 EST-SSRs assigned to the B genome of bread wheat were used to investigate the relationships between the species bearing B or related to B genomes. In this case, Jaccard genetic distance coefficients (Jaccard 1908) ranged from 0.074 between *T. aestivum* cv Chortandinka and Coppadra to 0.952 between *Ae. longissima* accession 1 and *T. aestivum* cv Aifeng. The data were bootstrapped to obtain a consensus tree from 100 UPGMA trees (Fig. 5). Similar to the previous study, tetraploid species were clustered together and were closely related to hexaploid species. *Ae. speltoides* accessions were grouped together while other sub-species were more dispersed. In this tree, *Ae. searsii* accession 4 was clustered with the polyploid wheats but in less than 60% of the UPGMA trees indicating that this linkage was not highly significant. However, this was in accordance with the results from Feldman (1978) who proposed *Ae. searsii* as a potential candidate for the B-genome donor. *Ae. speltoides* sub-species had also close relationships with the polyploid wheats which may suggest that both *Ae. searsii* and *Ae. speltoides* may have contributed to the elaboration of the B genome of polyploid wheats. A larger number of *Ae. searsii* accessions should be tested to confirm or reject this hypothesis.

In both cases, tetraploid and hexaploid wheats were found to be closely related but with no clear and significant splitting between them. This is probably due to the fact that they diverged only recently about 250,000 years ago.

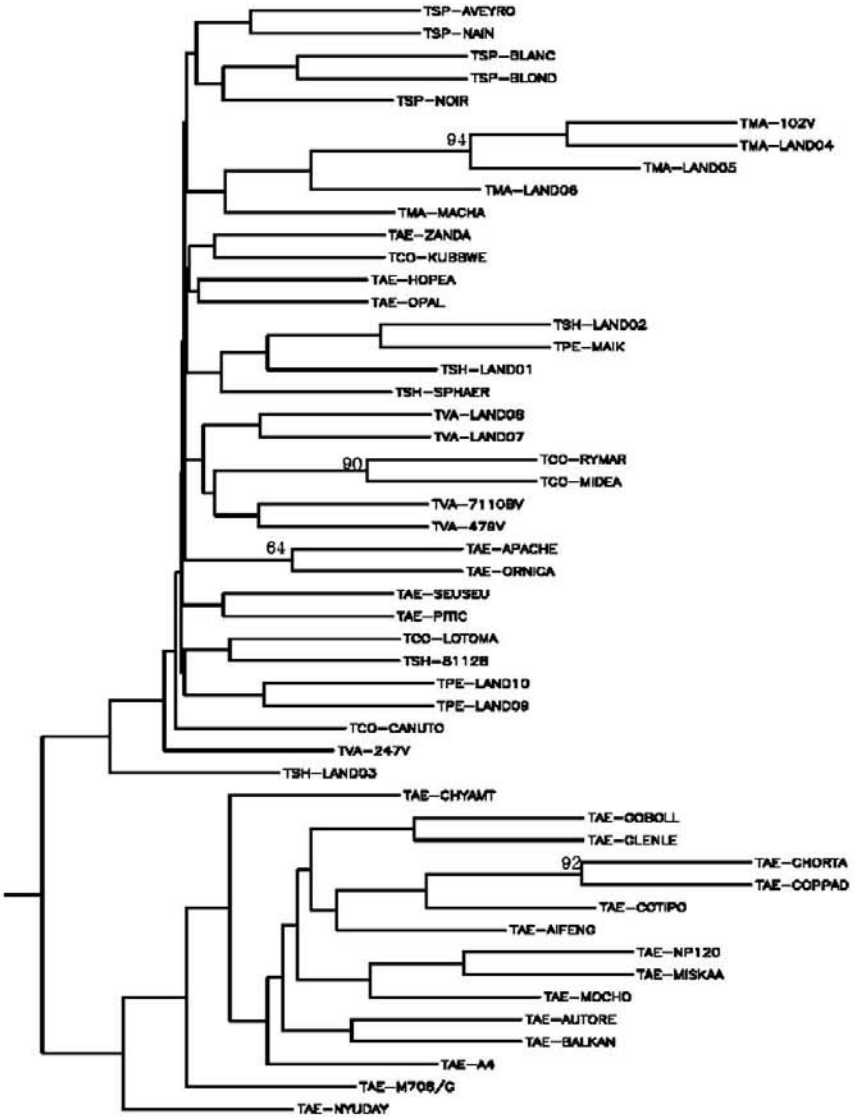

Fig. 3 Consensus tree of 50 accessions of hexaploid species reconstructed from 100 UPGMA trees obtained from data resampled in a set of 73 EST-SSRs. The accession codes are the same as those indicated in Supplementary Table 1 (TSP *T. spelta*, TMA *T. macha*, TAE *T. aestivum*, TCO *T. compactum*, TSH *T. sphaerococcum*, TVA *T. vavilovi*, TPE *T. petropavlovskyi*). The branch lengths are proportional to the number of times that each group appeared. Additionally, *numbers* indicated bootstrap values larger than 60%

Discussion

Bread wheat EST-SSRs were tested to explore their potential in phylogeny analyses of a large set of Triticeae species (14). We were thus able to compare the species according to their ploidy level (diploid species as well as tetra- and hexaploid species). We also compared the relationships between the diploid and the polyploid species according to the genome (A or B) using previously assigned EST-SSRs (Zhang et al. 2005).

Many methods exist for phylogenetic studies but there is a growing realization that the data on SSRs should be recorded as specific alleles in different genotypes and then used for analysis (Reif et al. 2005). This makes co-dominant SSRs better markers than other

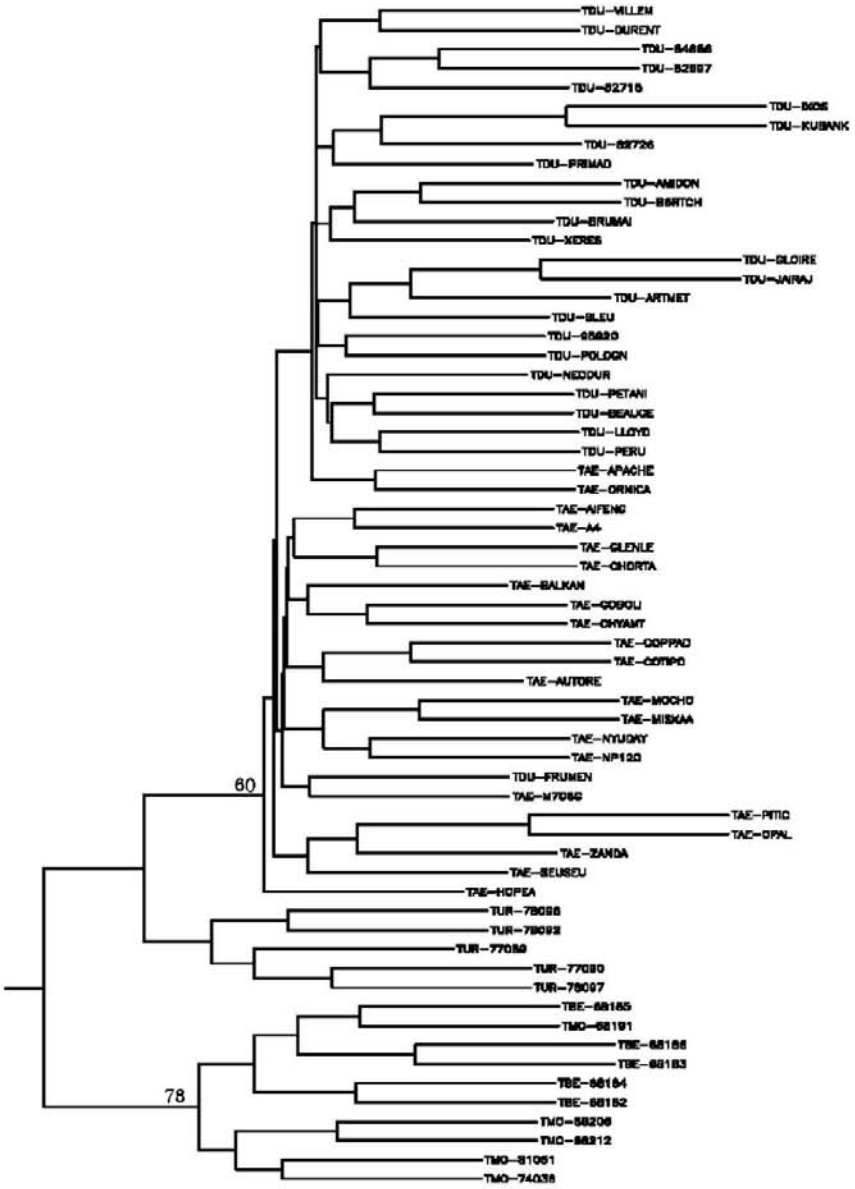

Fig. 4 Consensus tree of 62 Triticeae accessions (22 *T. aestivum*, 25 *T. durum*, 5 *T. monococcum*, 5 *T. urartu*, and 5 *T. boeoticum*) reconstructed from 100 UPGMA trees obtained from data resampled in a set of 16 EST-SSRs assigned to the A genome. The accession codes are the same as those indicated in Supplementary Table 1. The branch lengths are proportional to the number of times that each group appeared. Additionally, *numbers* indicated bootstrap values larger than 60%

dominant markers because if data are recorded as allelic variants, more powerful statistical analyses such as AMOVA or *F* statistics (Slatkin 1995) can be conducted. However, in this study, we analyzed a limited number of accessions for each species and because these species gave different number of alleles compared to wheat, the frequencies could not be estimated accurately. Moreover, some species were allogamous (*Lolium*, *Ae. speltoides*) and it was assumed that the diversity available within an accession was compensated by extracting DNA from a mix of several plants (see "Materials and methods"). It was thus inappropri-

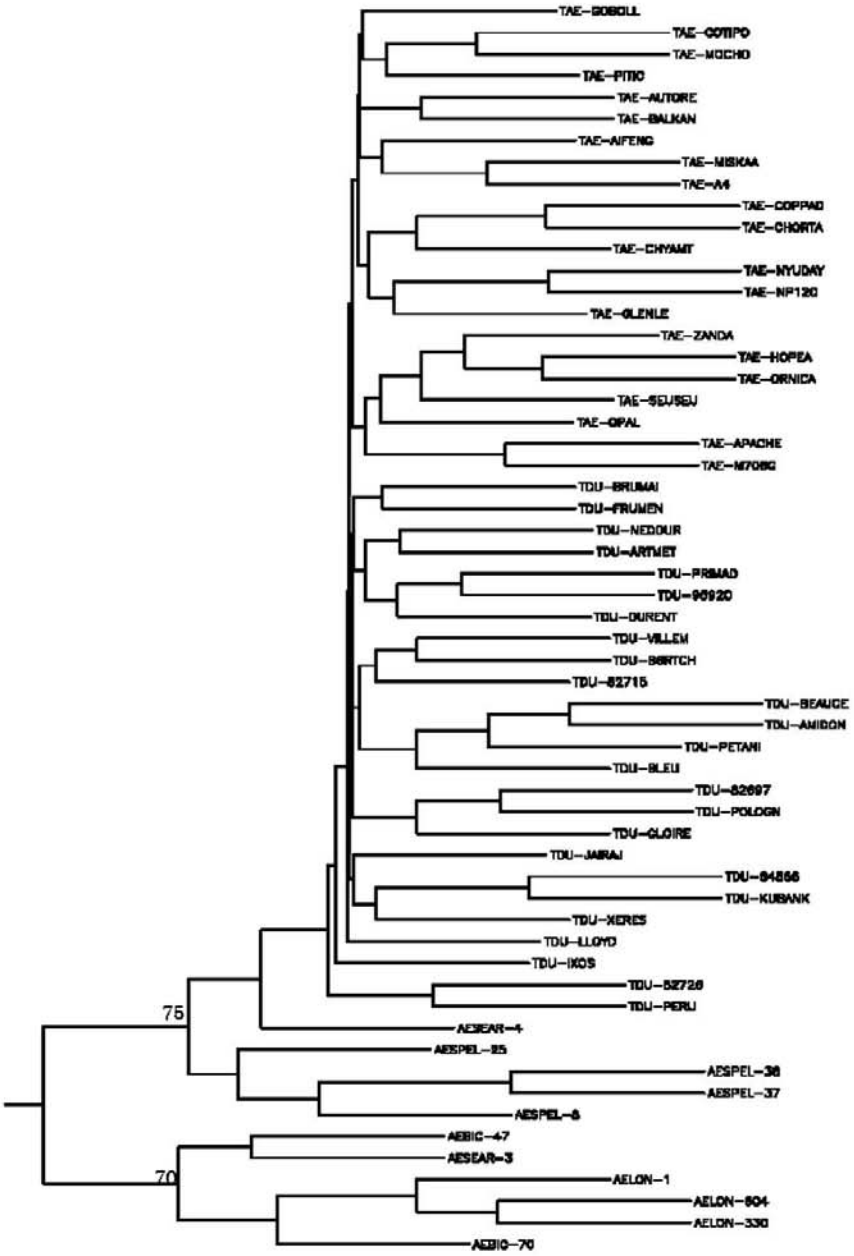

Fig. 5 Consensus tree of 58 Triticeae accessions (22 *T. aestivum*, 25 *T. durum*, 4 *Ae. speltoides*, 2 *Ae. searsii*, 2 *Ae. Bicornis*, and 3 *Ae. longissima*) reconstructed from 100 UPGMA trees obtained from data resampled in a set of ten EST-SSRs assigned to the B genome. The accession codes are the same as those indicated in Supplementary Table 1. The branch lengths are proportional to the number of times that each group appeared. Additionally, *numbers* indicated bootstrap values larger than 60%

ate to use AMOVA and *F* statistics in our case which led us to score the data on SSRs in a binary format (presence/absence of alleles). In this study, we used a phenetic method based on the Jaccard's distance matrix (Jaccard 1908). As the divergence between all the species studied was quite recent, the molecular

clock was assumed which led us to choose UPGMA clustering method rather than the NJ method (Saitou and Nei 1987). Under this assumption, dendrograms based on UPGMA can be discussed from a phylogenetic point of view. Similarly, the inadequacy of one marker system over a number of marker systems and morphological traits was also demonstrated (Gupta and Varshney 1999). In order to solve both problems (type of markers and analyses), it would thus be interesting to test a larger number of accessions from each species together with a larger number of EST-SSRs and compare the results obtained with other types of markers (gSSRs, RFLPs, AFLPs, SNPs) in order to know which type and how many markers and samples give the best results for phylogenetic studies among members of the Triticeae tribe.

When genetic similarity among diploid relatives of hexaploid wheat was investigated, most of the accessions of each species were clustered into their corresponding group in our dendrogram. The three groups of ancestral diploid species were clustered which was consistent with other taxonomic studies of the Triticeae tribe (Appels et al. 1989). The close associations observed between the B-genome and the D-genome donors were in agreement with previous studies using RFLP markers (Monte et al. 1993). The same authors showed that the ancestral diploid species were more closely related to *Hordeum* species compared with *Secale* species, which was also consistent with what we found. Regarding the B-genome possible donors, we tested four species which were divided into two distinct groups in our dendrogram. This was in accordance with previous studies which mentioned similar classifications (Ogihara and Tsunewaki 1988; Sasanuma et al. 1996, 2004), and this is also in agreement with the classification of the Sitopsis section into two subsections, one for *Ae. speltoides* alone and the other including *Ae. longissima*, *Ae. searsii*, *Ae. sharonensis* and *Ae. bicornis* (von Eig 1929). Concerning the discrepancies that we reported in our results, this suggests that we should reconsider the classification of some of our accessions.

The relationships between the tetraploid species were more confused. *T. durum*, *T. polonicum*, and *T. turgidum* sub-species were clustered together. This can be explained by the fact that these are three cultivated species or because not enough markers have been tested to clearly separate these species that diverged only recently. In addition, *polonicum* accessions were more or less dispersed among the *durum* accessions suggesting that they could have been involved in the pedigree of some current varieties. On the contrary, *carthlicum*, *dicoccum*, and *dicoccoides* sub-species were less related to cultivated accessions. The two former species were closer to all these tetraploid species while *dicoccoides* was found to be the less related, which confirms that this species could be the ancestor of all the tetraploid wheat species. The divergence could have occurred from *T. dicoccoides* to *dicoccum*, *carthlicum*, *turgidum*, and *durum*. This was consistent with the fact that the wild species *T. turgidum* ssp. *dicoccoides* was domesticated to form *T. t.* ssp. *dicoccum* and successive domestication steps generated durum wheat (*T. t.* ssp. *durum*), the most cultivated tetraploid wheat (Salamini et al. 2002). The origin and classification of *T. carthlicum* accession 94753, which undoubtedly roots the tree, should be confirmed prior to concluding on its position.

The hexaploid species were separated into two groups, one gathering most of the *aestivum* varieties, the other including the remaining sub-species and varieties. This latter group mainly contained European and Asian accessions indicating a possible common origin. In addition, the *aestivum* varieties from this group were dispersed throughout the cluster and were related to *compactum* as well as to *vavilovi* or *macha* accessions, suggesting that these species are either closely related or that they have been used as progenitors of wheat varieties in a course of European breeding programs.

In both tetraploid and hexaploid species, the difficulty in separating accurately the groups of sub-species can be explained by the high percentage of common bands between all the species, which probably reflect the high conservation of the coding sequences between all the Triticeae species. This problem would probably be solved by testing a larger number of either EST-SSRs or genomic SSRs. In this latter case, more diversity is supposed to be observed because most of genomic SSR markers detect polymorphism located in the non-coding regions of the genome which are supposed to evolve more rapidly (Brown et al. 2001). On the contrary, EST-SSRs derive from the expressed part of the genome and detect thus the genetic diversity appearing within the genes themselves. Using this strategy, Xu et al. (2004) reconstructed the phylogenetic tree for almond from China and the Mediterranean region. Two distinct groups were formed, one for Chinese cultivars and the other for the Mediterranean cultivars which agreed with their geographical origin. This suggests that a clear dendrogram based on a combination of both EST- and g-SSRs could be reconstructed for the wheat tetraploid and hexaploid species.

When the dendrogram was constructed based on data produced by a set of EST-SSRs assigned to the A genome of bread wheat, a clear phylogenetic relationship was obtained. All the polyploid wheats were

gathered in the same cluster and the relative positions of the tetraploid and hexaploid wheat accessions remained consistent to what was observed when each group was analyzed separately. This large group was more closely related to the group including *T. urartu* (A^uA^u) sub-species while the other A-genome diploid species [*T. monococcum* (A^mA^m) and *T. boeoticum* (A^bA^b)] were clustered together and were less related to polyploid wheats. Earlier cytogenetic studies suggested that the A genome of common wheat was contributed by *T. monococcum* (Sax 1922; Lilienfeld and Kihara 1934) but more recent evidence showed that *T. urartu* contributed the A genome of hexaploid wheat (Dvorak et al. 1993; Huang et al. 2002). Our results support the latter point of view.

Similarly, as for the analysis using A-genome EST-SSRs, the polyploid wheats were consistently clustered together using B-genome EST-SSRs while fewer markers were used (10 instead of 16 for the A-genome markers). This may be due to the fact that the B genome is frequently more polymorphic than the other two (A and D) and the markers that are located on this genome are thus more appropriate to separate the different species. For the origin of the B genome of polyploid wheats, our results indicate that *T. aestivum* and *T. durum* are more closely related to *Ae. searsii* accession 4 and *Ae. speltoides* (SS) sub-species than with other species bearing the S genome [*Ae. searsii* accession 3 (S^sS^s), *Ae. bicornis* (S^bS^b), *Ae. longissima* (S^lS^l)]. The origin of the B genome has been the subject of considerable speculations and investigations and it still remains largely unresolved. Earlier studies proposed lots of possible donors in the large range of the Poaceae family (Miller 1990), whereas other experiments have focused on *Ae. speltoides* (Natarajan and Sharma 1974; Chen et al. 1975; Jaaska 1980; Hassan and Gustafson 1996; Maestra and Naranjo 1998). Our results suggest that among the species bearing the S genome, *Ae. speltoides* and *Ae searsii* sub-species have probably largely contributed to the B genome of polyploid wheats, which supports its polyphyletic origin.

In conclusion, we have shown that common wheat EST-SSRs can be useful for phylogenetic studies in the Triticeae tribe especially between distant species such as rye, barley, and wheat. They can also be used in elaborating dendrograms agreeing with those obtained using data from morphological, physiological, RFLP, and nuclear sequences. It would now be interesting to scale up the analysis by increasing the number of accessions within each species as well as the number of markers tested to have more accurate data on the Triticeae phylogeny.

Acknowledgments The authors gratefully thank G. Gay and A. Loussert for growing the plants and J. David for providing with *T. turgidum* sub-species. G. Boutet and C. Pont are also greatly acknowledged for their help in managing the robotics of the genotyping platform. This work was funded by the China Scholarship Council (CSC). All these experiments comply with the current laws of France.

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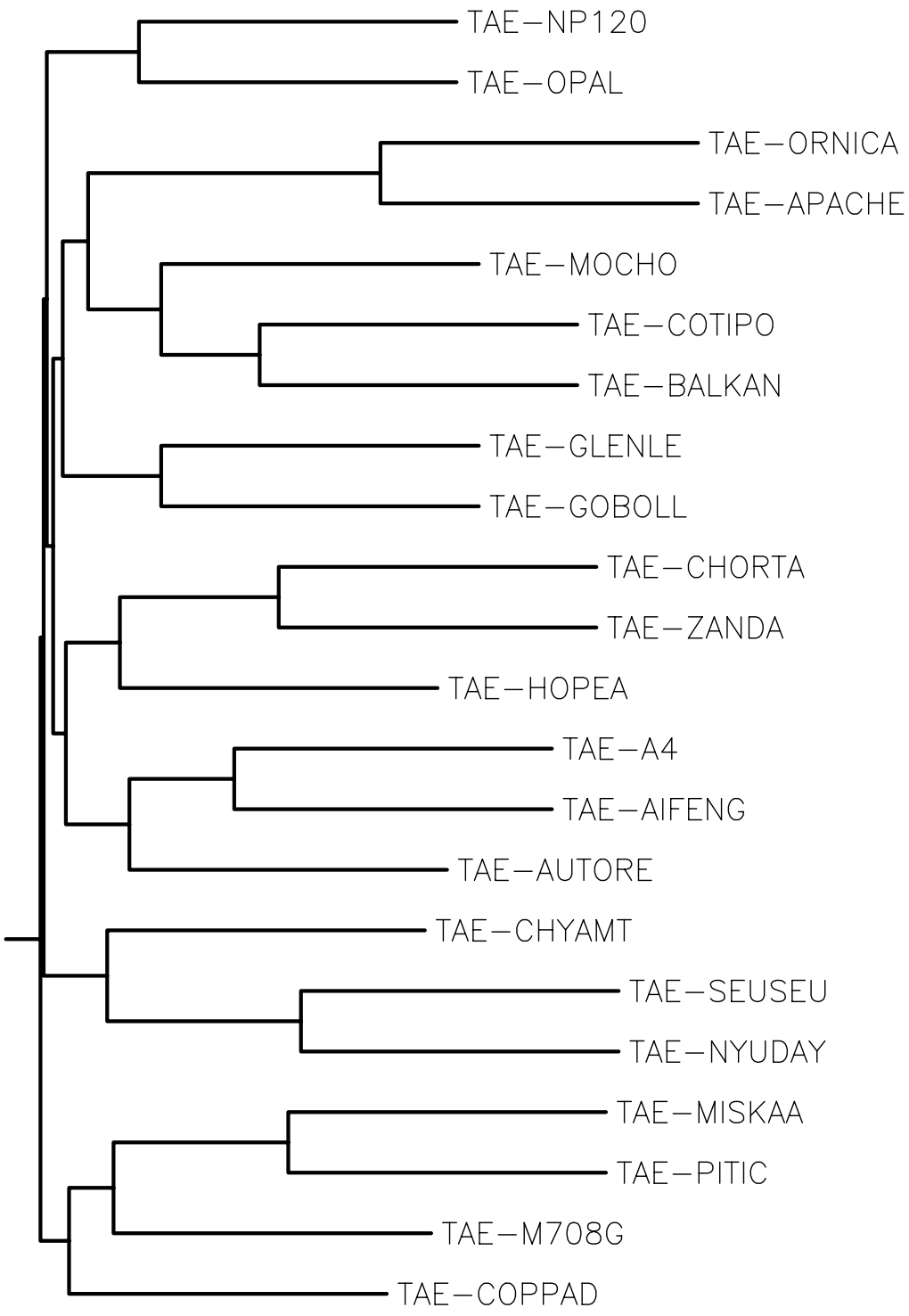

Figure 3-7: Dendrogram of 22 *T. aesticum* genotypes based on UPGMA method using 39 SSRs polymorphisms.

4.2 Comparison of the classification of *T. aestivum* lines obtained using g-SSRs and EST-SSRs

We decided to compare phylogenetic trees of 22 bread wheat cultivars reconstructed using 39 g-SSRs distributed on all of the 21 chromosomes of bread wheat (Roussel et al, 2002), and a similar set of 42 EST-SSRs.

For the 39 g-SSRs, 368 informative fragments were obtained and the Jaccard (1908) genetic distance coefficients ranged from 0.7884 between cvs Ornicar and Apache to 0.9863 between cvs Chortandinka and M708g. For the 42 EST-SSRs, 206 informative fragments were obtained and the coefficients ranged from 0.2571 between cvs A4 and Np120 to 0.6024 between cvs Chyamtang and Ornicar. These results clearly show that the g-SSRs are able to reveal a larger diversity compare to EST-SSRs, which are obviously less polymorphic because located in coding sequences.

In the following section, in each case the consensus dendrograms summarized 100 phylogenetic UPGMA-trees obtained from bootstrapped data.

In the dendrogram reconstructed from g-SSR data (Fig 3-7), no clear clustering was obtained and most of the bootstrap values were lower than 60%. However, there is a trend in forming five groups. The first one is made of only two lines from India (NP120) and the Netherlands (Opal). The second one contains five European lines (Ornicar, Apache, Mocho, Balkan, Gobolloi) together with a Canadian line (Glenlea) and a Brasilian line (Cotipora). The third group can be divided into two sub-groups, one with European lines (Chortandinka, Zanda, Hopea) and the other with Asian lines (A4, Aifeng, Aurore). Three Asian cultivars (Chyamtang, Seuseun, Nyuday) formed the fourth group while three Middle Eastern cultivars (Miskaagani, M708G, Coppadra) and the Mexican line (Pitic) clustered together to form the fifth group. Globally, the lines from the same geographic region tend to cluster together suggesting a spatial organization.

Similarly, no clear clustering was identified in the tree reconstructed from EST-SSR data (Fig 3-8). A large group contains all the lines except Nyuday (Japan) and Chyamtang (Nepal). Within the large group, sub-groups were obtained but they gathered lines from different origins except the one with Ornicar, Apache (Fra), Opal (Netherlands) and Hopea (Finland) which included only European lines.

We combined the data collected from g-SSRs and EST-SSRs to make a new dendrogram. Overall, 574 informative fragments were used to calculate Jaccard (1908) genetic distance

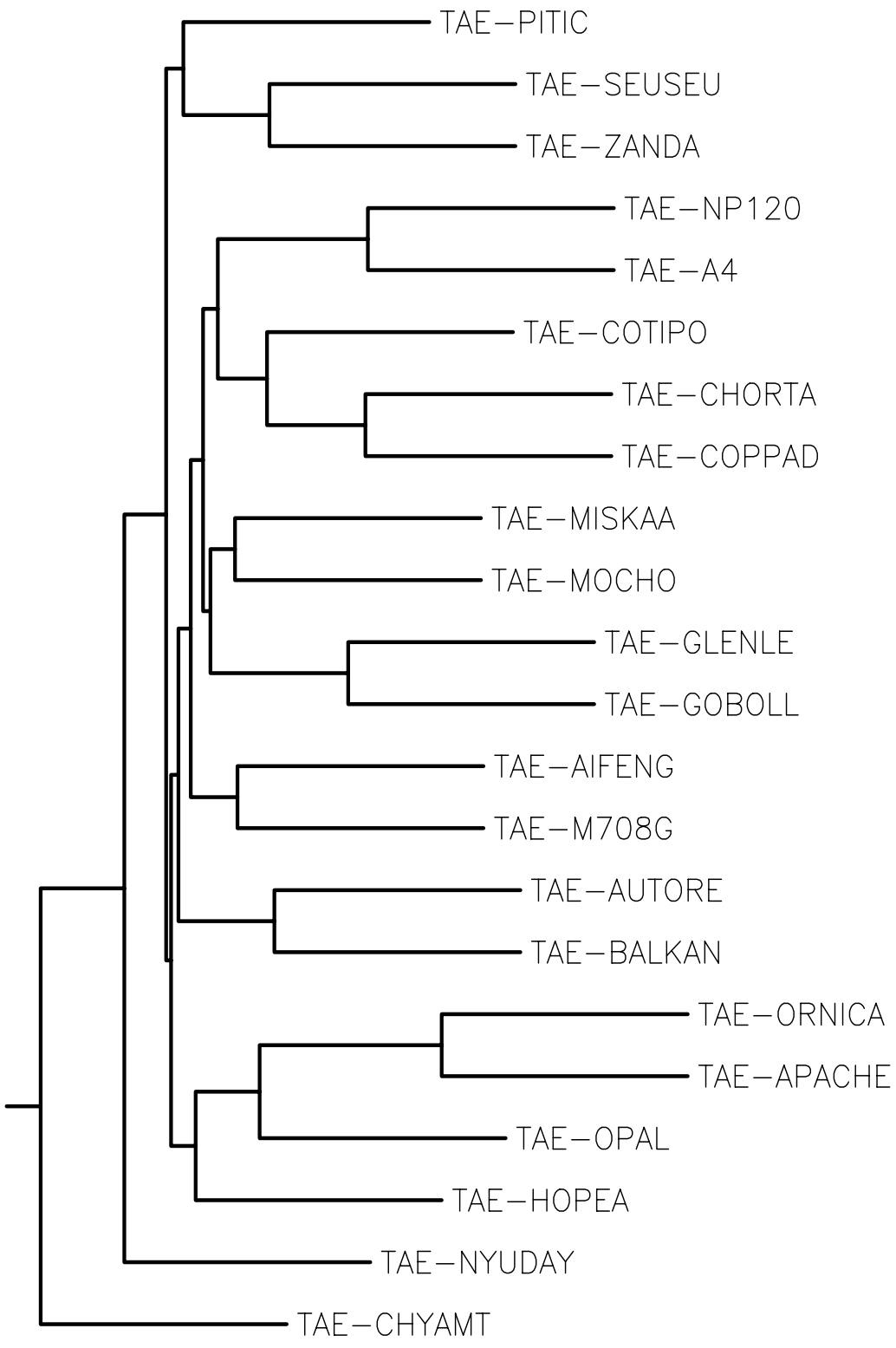

Figure3-8: Dendrogram of 22 *T. aesticum* genotypes based on UPGMA method using 42 EST-SSRs polymorphisms.

coefficients which ranged from 0.4596 between cvs Ornicar and Apache to 0.7482 between cvs A4 and Pitic. By pooling data from the two types of SSRs, a new dendrogram was rebuilt for the 22 accessions of *T. aestivum* (Fig 3-9). Like for the two other trees, no clear clustering was obtained. Similarly as for EST-SSRs, a large group containing most of lines was obtained, only Nyuday (Japan) and Chyamtang (Nepal) being excluded. Within the large group, French cultivars (Apache, Ornicar) were separated and grouped together. Cultivars Aifeng (China) and A4 (Afghanistan) were associated as well as the cvs Cotipora (Brasil) and Mocho (Portugal). The other lines with possible common origins were distributed in all the other sub-groups.

Comparing the two trees obtained using g- and EST-SSRs, the values of genetic distance (GD) were considerably different. The GD values obtained with EST-SSRs were lower (mean GD of 0.43) than those with g-SSRs (mean GD of 0.89) probably because the regions detected by EST-SSRs are well conserved among species. Concerning the tree reconstructed based on pooled data, GD values were a mean of those for EST- and g-SSRs (mean GD of 0.6), and new relationships were observed. The three dendrograms were significantly different and no clear conclusion on a better capacity of one or the other type of marker to discriminate between the lines was drawn.

4.3 Genetic diversity of Triticale species

The genetic diversity of eight Triticale varieties was also investigated by using wheat EST-SSRs. Polymorphism information content (PIC) values which provide an estimate of the discriminatory power of each EST-SSR locus were computed using 47 EST-SSRs. In this case, EST-SSRs were chosen according to their ability to reveal polymorphism and to the absence of missing data. PIC values ranged from 0.23 to 0.77 (average 0.41 ± 0.17) which was similar to those obtained for *T. aestivum* (average 0.40 ± 0.20) and for *T. durum* (average 0.39 ± 0.19). This value was lower than that of g-SSRs (mean value of 0.54), reported by Tams et al. (2004).

For the 47 EST-SSRs, 185 informative fragments were obtained. The Jaccard genetic distance coefficients were calculated using these data and ranged from 0.25 between cvs Trimaran and Tricolor to 0.65 between cvs Lamberto and Bienvenu. The consensus dendrogram was reconstructed by using UPGMA method (Fig. 3-10). In this tree, the varieties were clustered according to their geographic origin (France and Poland). Within the French cultivars, two groups were formed, one where four accessions were clustered

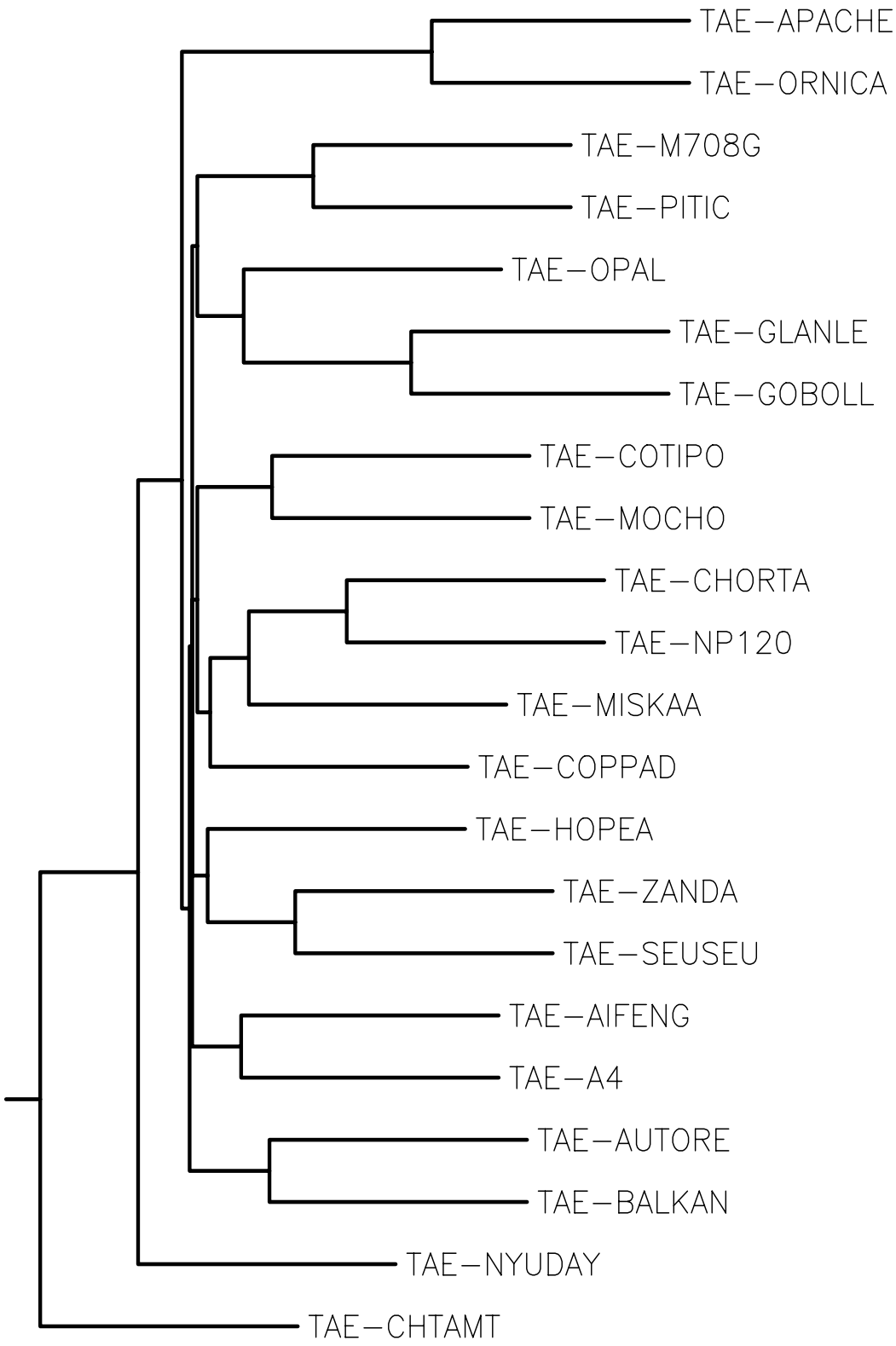

Figure 3-9: Integrated dendrogram of 22 *T. aesticum* genotypes based on UPGMA method pooled data from 42 EST-SSRs and 39 g-SSRs polymorphisms.

together (Zeus, Ampiac, Bienvenu and Matinal), the other with the remaining three (Trimaran, Tricolor and Carnac). All these results were consistent with the known pedigree of the lines (A Bouguennec, personnal communication). Our result demonstrated thus that common wheat EST-SSRs can be successfully used for diversity estimation in relatives of wheat.

4.4 Phylogenetic relationship for species with the D genome

In order to confirm the relationship between *Ae. tauschii* and polyploid wheats, one set of ten markers evenly distributed on the D genome of hexaploid wheat (Zhang et al. 2005) was selected to investigate the relationships between this genome of hexaploid, tetraploid and *Ae. tauschii* species.

In this case, 169 informative fragments obtained from 10 EST-SSRs assigned to the D genome of bread wheat were used. The Jaccard genetic distance coefficients (1908) were calculated using these data and ranged from 0.2105 between *T. aestivum* cvs NP120 and Chyamtang to 0.9615 between *Ae. tauschii* accession 33 and *T. durum* cv Peru1. The consensus dendrogram which summarized 100 phylogenetic UPGMA-trees obtained from bootstrapped data is shown in Fig. 3-11.

Two distinct groups were formed. Hexaploid wheats (ABD) and *Ae. tauschii* (D) species were clustered together and formed one large group because both have a D genome originating from a common ancestor. All *Ae. tauschii* accessions were grouped together, accession 42 being slightly isolated from all the other accessions. A close relationship was shown between accessions 13 and 32, and between accessions 15 and 33. Since tetraploid species (AB) have no D genome, they clustered together to form another group.

4.5 Phylogenetic relationships between all the species

In order to examine the phylogenetic relationships among all the 32 species or sub-species, the most informative accession (with as fewer missing data as possible) of each group among all those tested was selected. They are indicated in annex 2. A set of 73 EST-SSRs was selected and the Jaccard genetic distance coefficients were calculated based on the 867 informative fragments. The GD coefficients ranged from 0.224 between *T. sphaerococcoccum* and *T. petropavlovskyi* to 1.00 between *T. polonicum* and *Oryza sativa*. A dendrogram was reconstructed, which summarized 100 phylogenetic UPGMA-trees obtained from bootstrapped data (Figure 3-12). In this phylogenetic tree, all the subspecies with the same genome were clustered together without ambiguity. The hexaploid

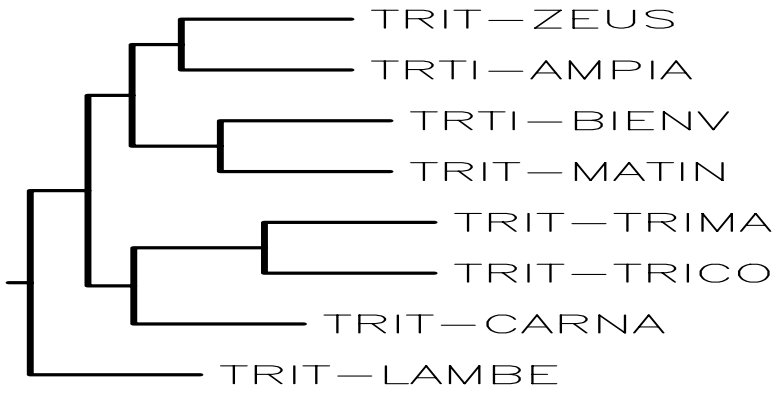

Figure 3-10 Dendrogram of 8 *Tritical* genotypes based on UPGMA method using data based on 47 EST-SSRs.

(AABBDD) and tetraploid wheat (AABB) were grouped together with the boostrapped values of 100. *Ae. tauschii*, the D genome donor of bread wheat was related to *Ae. ventricosa* (DDM^vM^v) which also carries a D genome. Among the diploid ancestral species, *Ae. tauschii* was the most closely relative to hexaploid wheats followed by the A- (*T. urartu* and further *T. monococcum* and *T. boeoticum*) and the B-genome (*Ae. speltoides* and *Ae. searsii* and further *Ae. bicornis and Ae. longissima*) donors respectively. When more distant species are considered, *Avena sativa* and *Hordeum vulgare* were closer to hexaploid wheat whereas *Oryza sativa* and *Lolium perenne* appeared to be the most distant species.
In the hexaploid group, *T. sphaerococcum, T. petropavlovskyi, T. vavilovi* and *T. spelta* were clustered together while *T. compactum, T. macha* and *T. aestivum* were separated from this group and from each other. In the tetraploid species, *T. durum, T. polonicum* and *T. turgidum* were clustered together, and the remaining species (*T. carthlicum*, *T. dicoccum*) were separated from each other. Triticale was clustered into the tetraploid group which was consistent with the fact that the cultivars tested were all hexaploids and only had the A and B genomes of polyploid wheats. The S genome species were split into two groups, one with *Ae. bicornis* (S^bS^b) and *Ae. longissima* (S^lS^l) and the other with *Ae. speltoides* (SS) and *Ae. searsii* (S^sS^s). *Ae. umbellulata* (UU) and *Ae. peregrina* (UUS^vS^v) were clustered with the S-genome species indicating close relationships between the U and S genomes.

4.6 Discussion

As expected, EST-SSRs revealed a lower number of alleles compare to gSSRs. This is because they arise from coding regions, which are known to evolve slower compare to non-coding regions. Similarly, the tree obtained using g-SSRs gave a better representation of the relationships between the lines compared to the one issued from EST-SSRs. Because g-SSRs are random genomic DNA markers, most of them detect polymorphism located in the non-coding regions of the genome (Brown et al. 2001). They are thus less constrained by selection, can exhibit a higher number of alleles and are thus more informative (Roussel et al. 2004, 2005). This suggests that more EST-SSRs are needed to achieve the same resolution than the g-SSRs. Gupta et al. (2003) reported similar results on wheat. They assessed the degree of genetic diversity among 52 elite wheat genotypes by using 78 wheat EST-SSRs. They recommended the use of one or more of the other available marker systems (for example g-SSRs) together with EST-SSRs. Thereby, the genetic diversity, based on data pooled by combining EST-SSRs and g-SSRs, will give more reliable patterns,

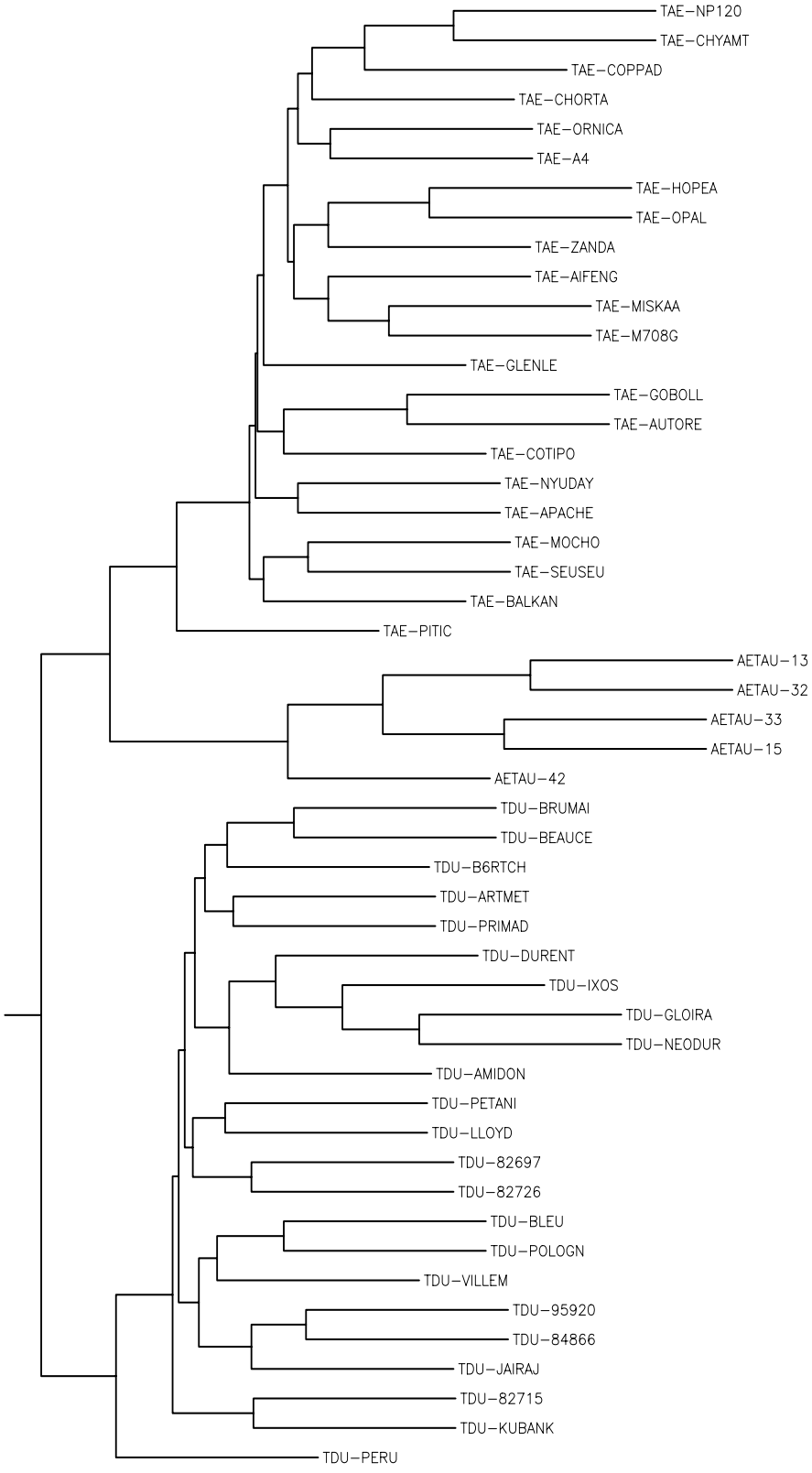

Figure 3-11 Dendrogram for 58 accessions (22 *T. aestivum*, 23 *T. durum*, 5 *Ae. tauschii*,) reconstructed using the UPGMA method based on data of a set of 10 EST-SSRs assigned to the D genome.

since these will be based on a relatively much larger proportion of the entire genome.

Triticale (x*Triticosecal* Wittm), an amphiploid species derived from the intergeneric hybrid between wheat and rye, has gained considerable importance in recent years as a cattle feed grain, due to its favorable amino acid composition and performance in less productive environments. From our investigations, we found that the seven French cultivars we analysed were split into two groups. This can be explained by the origins of these cultivars. Varieties Trimaran, Carnac and Tricolor were breeded by Florimond-Desprez, and have a common ancestor in their pedigree which explain the clustering of these three lines (A Bouguennec, personnal communication). Similarly the other four varieties have also common ancestors. The results are thus consistent with the known pedigree of the lines.

Our study also gave evidence that *Ae. tauschii* was the D-genome donor of hexaploid wheat. Dvorak et al. (1998) used the variation at 27 RFLP loci to determine the genetic distance between wheat and *Aegilops* and found 16 alleles shared by the two species. Data presented in their studies pointed to subsp. *strangulate* as the source of the wheat D genome. This finding supports previous investigation using isozymes, and HMW glutenin subunits (Nakai 1978; Lagudah et al. 1991). However, using microsatellites, Lelley et al. (2000) did not support this hypothesis and the problem of which specific genotype of *Ae. tauschii* was the major source of the wheat genome still remains unresolved.

Concerning the tree constructed for all species, hexaploid, tetraploid, Triticale and D-genome donor species were grouped with a better consistency compared to the remaining species (the A and B genome donors, and wild relatives of common wheat). This may be attributed to the method used for selecting the samples and the low number of data points (867) used. However, in this tree, the hexaploid and tetraploid species showed a very close relationship that was consistent with the phylogeny of the polyploid wheats. They also appeared to be closer to the D- (*Ae. tauschii)* and A-genome donor species than to the B-genome donor species. This result agreed with those reported by Buchner et al. (2004) and Galili et al. (2000) based on RFLP analysis and with those from Zhang et al. (2002) based on DNA sequence analysis.

Early cytogenetic studies suggested that the A genome of common wheat was contributed by *T. monococcum* (Sax 1922; Lilienfeld and Kihara 1934). More recent studies showed that *T. urartu* contributed the A genome to hexaploid wheat (Dvorak et al 1993; Huang et al 2002). In our phylogenetic trees, *T. urartu* also showed a closer relationship with hexaploid wheat within the three possible A genome donors. Our results thus confirmed the

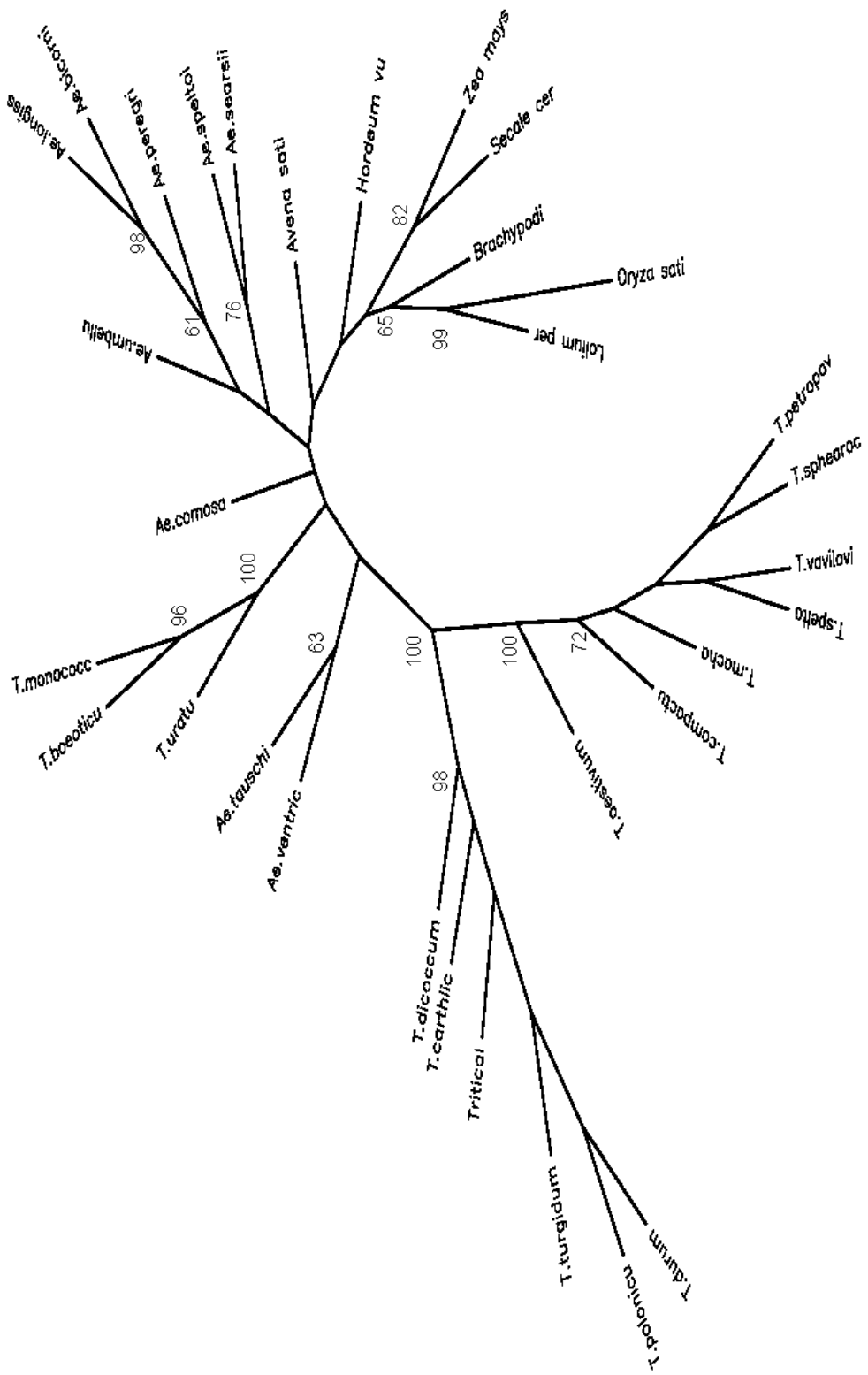

Figure 3-12 Dendrograms for 32 representatives from 32 species reconstructed by UPGMA method. Numbers on the branche indicate the bootstrap values for 100 replicates. Values greater than 60% are shown

known phylogenetic relationship among common wheat and its ancestral species, *Ae. tauschii* was the likely progenitor of the D genome and *T. urartu* the one of the A genome. Some discrepancies were observed with phylogenetic trees reconstructed based on cytoplasmic genomes which allows to trace the female donor of present cytoplasm (Ishii et al. 2001; Wang et al. 1997) suggesting that the evolution was not parallel between the cytoplasmic and the nuclear genomes in *Aegilops* (Sasanuma et al. 2004).

4.7 Comments and perspectives

As a conclusion, bread wheat EST-SSRs showed that they can be useful for estimating intraspecific genetic diversity. However, they give better results when they are combined with g-SSRs. A fair comparison might have been to estimate the relationships either within or between species on a given set of lines starting from many EST-SSRs as well as g-SSRs. Then an estimate of the efficient number of SSRs (in each case) needed to achieve a given precision of the GD/GS would have been obtained by resampling the data.

EST-SSRs are also proved useful for taxonomic and phylogenetic studies even between distant species such as rice, maize, rye, barley and wheat. Trully speaking, we did not assess the genetic diversity but only the relative classification of the accessions. However, better information would be obtained if sequences of the amplification products were analyzed. We would then access to the SSR itself and to the variations in terms of type of motif, number of repeats, point mutation within and outside the SSR.

GENERAL CONCLUSION

General Conclusion

1. Analysis of the wheat ESTs

1.1Main results

Among the 301 wheat EST-derived SSRs selected, the trinucleotide repeats were the most common (77%), (GCC)n motif being the most abundant and SSRs with four repeats occurring the most frequently.

About 80% of the primer pairs gave an amplification product with some differences between their expected and observed sizes. In general, EST-SSRs markers produced high quality patterns compare to g-SSRs. Dinucleotide repeats displayed higher level of polymorphism than trinucleotide repeats. The combination W7984 x Opata (37.5%) was the most polymorphic, the average level of polymorphism being 25.4% for the four populations. Most of the EST-SSRs gave less than five amplification products using our sample, one third giving three bands probably corresponding to the three homoeologous chromosomes while 50 % gave only one band suggesting that these latter were specific of one of the three copies or that the three copies had identical sizes or that these SSRs were located within unique genes.

Overall, 81 (62 on ITMI reference map and 19 on the CtCS map) new loci were added to the wheat genetic maps. More loci mapped to the B genome compared to the A and D genomes confirming that the former exhibits more polymorphism than the other two. Only few loci mapped to group 5 chromosomes suggesting a better conservation and less polymorphism within the genes from these chromosomes compared to the others. Most of the loci located in the distal regions of chromosomes, which was consistent with the known location of genes in wheat.

1.2Perspectives

According to our results, developing new markers from ESTs can be a good way to improve rapidly the wheat genetic map with non anonymous markers. However, we must be aware that (1) the primers should be designed accurately in order to avoid to be at a junction between or on two different exons. In this case, no useful amplification will occur.

One way to overcome this would be to use the rice sequence since there is a good conservation of the splicing junction between the two species; (2) the level of polymorphism will be low which will make them difficult to map. This problem can be overcome by combining both SSR length and sequence variation using SSCP analysis that is able to discriminate between two strands differing by only a limited number of base pair changes or other methods. Since SSRs are known to evolve faster than other type of sequences, it is likely that they show higher levels of mutations, even within coding sequences; (3) the markers that are going to be developed will be located in the distal ends of the chromosomes. However, this is maybe not a problem since markers are mainly developed to identify and follow genes of agronomical interest. Most of these genes are also located distally on the chromosomes. Therefore, developing markers in these regions will be helpful for identifying linkage with QTLs and for further positional cloning of the genes underlying these QTLs.

In order to answer the questions concerning the various numbers of bands, it would be interesting to confirm the presence of the three homoeologous copies by doing some hybridization using the EST as a probe when only one amplification product is observed. For those giving several bands, we could also sequence the different copies in order to compare the sequences and study the differences concerning the SSR between them.

2. Transferability of the wheat EST-SSRs

2.1Main results

Transferability to closely related *Triticeae* species ranged from 73.7% for *Ae. longissima* to 100% for some wheat sub species (*T. compactum*) and was lower for less close relatives such as barley (50.4%) or rice (28.3%). BLAST analysis of the EST sequences against the twelve rice pseudo-molecules showed that the EST-SSRs are mainly located in the telomeric regions, and that the wheat ESTs have highest similarity to genes on rice chromosomes 2, 3 and 5. Interestingly, most of the SSRs giving an amplification product on barley or rice had a similar repeated motif as the one found in wheat suggesting a common ancestral origin.

On average, the number of alleles per locus detected by EST-SSR markers was 3.1 on our sample of hexaploid wheats. The PIC values simultaneously estimated for *T. aestivum* and

T. durum were similar for the two species (0.40 and 0.39 respectively). The allelic diversity within species revealed that allogamous species such as *Ae. speltoides, Lolium perenne* and maize showed the highest values (respectively 0.423, 0.388 and 0.352) suggesting higher level of genetic diversity within these species compare to *T. aestivum* and *T. durum* which exhibited the lowest values (0.108 and 0.093 respectively). *T. aestivum* and *T. durum* shared the largest number of alleles (74.6%) while among the three ancestral diploid species of bread wheat, *Ae. tauschii* had the highest percentage of common alleles with *T. aestivum* (57.4%), followed by *T. monococcum* (47.6%) and *Ae. speltoides* (40.9%).

2.2Perspectives

Our results indicate that wheat EST-SSRs have a good potential for genetic map elaboration in orphan species. Despite the fact that these latter have a great interest for numerous traits they are only poorly studied because they carry many undesirable characters. If favorable alleles can be identified in these genotypes, existence of linked molecular markers will be necessary to trail their introgression within elite germplasm. However, because no genomic tools have been developed for these species and especially molecular markers, such a marker assisted selection was not possible. EST-SSRs that are transferable across the species will allow overcoming of this drawback. Introgressions could thus be reduced to a minimum, avoiding the linkage drag of large fragments of alien chromosomes bearing numerous undesired genes (forward and backward selection). Moreover, because of the large genetic diversity existing in wild species, there could be a simultaneous improvement of the bread wheat genetic diversity that has tended to decrease dramatically since the last 40 years.

3. Phylogenetic studies

3.1Main results

When diploid species only are concerned, all the accessions bearing the same genome were clustered together without ambiguity while the separation between the different sub-species of tetraploid as well as hexaploid wheats was less clear. Phylogenetic trees reconstructed based on data of 16 EST-SSRs mapping on the A genome showed that *T. aestivum* and *T. durum* had closer relationships with *T. urartu* than with *T. monococcum* and *T. boeoticum*, confirming that *T. urartu* is the A genome ancestor of polyploid wheats. Similarly, another tree reconstructed from data of 10 EST-SSRs mapping on the B genome

showed that *Ae. speltoides* had a closest relationship with *T. aestivum* and *T. durum*, suggesting that it was the main contributor of the B genome of polyploid wheats.

No clear clustering of the hexaploid varieties was identified in the tree reconstructed from EST-SSR data and the results were better using g-SSRs. It was not possible to compare the two trees because they were too different from each other. Result was better with Triticale where varieties could be separated according to their origin. Similarly, studying the hexaploid and tetraploid species together with *Ae. tauschii* species using D-genome EST-SSRs made easier the separation in different groups. Moreover, when all the 32 species or sub-species are analyzed together, the clustering is much better and the phylogeny between all the species seems to appear more clearly.

3.2Perspectives

In this study, we used only a limited number of markers (16 and 10 for A and B genome analyses respectively) which make our classification not highly statistically significant. However, we showed that EST-SSRs constitute a good tool for phylogenetic studies within the Grass family. We have some evidence that when amplification occurs in different species, the microsatellite motif is often present in all the species and sometimes similar between the species even if the number of repeats is not the same. However, a better study would consist in amplifying in as many species as possible a significant number of EST-SSRs and sequencing the amplification products. Then, we could do a more significant phylogenetic study of the relationships between all the species by comparing homologous sequences of the same gene. It would then be possible to draw a scheme of the molecular evolution of all these species on a sufficient number of sequences and reconsider or confirm the classification within the Grass family.

4. Transferability of rice EST-SSRs

4.1 Main results

Rice as a model for grass species has been largely studied to understand other agronomically important grass genomes. Here, the diversity of SSRs on rice chromosome 1 was surveyed, and primer pairs were developed to explore the colinearity between rice and wheat, and to investigate their potential for comparative genomics. A total of 12,078 perfect as well as imperfect SSRs were identified from 4,100 genes containing SSRs on

rice chromosome 1. Like in wheat, the trinucleotide repeats were the most common in rice chromosome 1 genes (whole genes, CDS and introns) (CCG)n motif being the most abundant in the three cases. (AC)n dinucleotide motif was most abundant in whole gene and in intron while CG was more frequent in CDS. 106 EST-SSRs were developed from CDS from 200 genes. 96% (102) of the primer pairs led to an amplification product in rice among which 96% (98) yielded a product with an expected size. The level of transferability from rice to eight related species of wheat (*T. aestivum, T. durum, T. monococcum, Ae. speltoides, Ae. tauschii,* rye (*Secale cereale*), barley (*Hordeum vulgare* L.), *Agropyron elongatum*) ranged from 28.4% (*T. aestivum*) to 6.9% (*Agroprum elongatum*). Sixteen markers were assigned to wheat chromosomes using NT and DT lines among which half of them were located on the wheat homoeologous group 3 suggesting that micro-colinearity is quite well conserved between wheat and rice in this region.

4.2 Perspectives

Our results show that due to their conservation and high transferability to the related species, rice EST–SSRs will be useful for comparative genomics studies. However, because the transferable EST-SSRs are better conserved, they are less prone to detect polymorphism and can thus be difficultly mapped. This problem can be hurdled by combining both SSR length and sequence variation using SSCP analysis. Though the structural syntenic relationships between wheat and rice at the macro level were demonstrated, the fact that only half of markers were assigned to wheat homoeologous group 3 suggests the complexity of micro-colinearity between rice and *Triticeae*. However, available rice genomic sequences can serve as excellent resources for the saturation mapping of the target region containing agronomically important genes with gene-based markers (such as EST-SSRs, etc), and finally may facilitate synteny-based positional cloning of gene from large genome cereal species

5. Conclusion

As a conclusion, we can say that it should be worth continuing the development of EST-SSRs in wheat which will be helpful for numerous genetic studies as well as the identification and cloning of genes of agronomical interest. Based on syntenic relationships between Triticeae and rice, the rice genome sequence can also serve as excellent resources for the saturation mapping of the target regions containing agronomically important genes

through a comparative approach, and finally may facilitate synteny-based positional cloning of genes within large genomes of cereal species, such as bread wheat. EST-SSRs can exhibit perfect marker-trait association, and can remarkably increase selection efficiency in application of marker-assisted selection (MAS), particularly for traits with low heritability. In addition, these markers will greatly help the exploration and exploitation of the genetic diversity existing among the Triticeae. They will probably constitute a breakthrough in wheat breeding by bringing a new vision of the way to increase the diversity and the sustainability of the crop which will lead to a better management of the natural resources such as water and a lower utilization of intrans.

Moreover, numerous questions remain to be answered:

- Despite the huge number of EST sequences, it has still not been evidenced a clear correlation between certain classes of genes and the presence of the SSR. Especially, the multicopy families have never been studied and it could be interesting to evaluate the different copies in order to see if all of them have the same SSR and if it has evolved differently between the copies.

- How can the SSRs be maintained or supressed in the different homoeologous copies? Is this randomly done or does this depend on the place of the SSR within the sequence?

- When the SSR is common to different species, this is because it has a common ancestral origin. However, we have no idea if newly synthetized SSR can occur, which are the mechanisms of this occurrence, are they genome or species specific, etc.

It was generally admitted that because of the low transferability, SSRs cannot be used for comparative genomics and synteny. However, EST-SSRs have proven the contrary. Studying the evolution of the SSRs (motif, number of repeats, point mutations…) within the grass family will probably lead to modify and reconsider the classification made on phenotypic traits. This will also probably contribute to understand the origin of the B genome of the bread wheat which still remains unexplained. Probably its polyphyletic origin would be confirmed by identifying the various species that may have contributed to its elaboration.

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ANNEXES

Annex 1

Fifteen international agricultural research centres supported by the CGIAR

Annex 1

Fifteen international agriculture Research centres:

Africa Rice Center (WARDA)

CIAT - Centro Internacional de Agricultura Tropical

CIFOR – Center for International Forestry Research

CIMMYT – Centro Internacional de Mejoramiento de Maiz y Trigo

CIP – Centro Internacional de la Papa

ICARDA – International Center for Agricultural Research in the Dry Areas

ICRISAT – International Crops Research Institute for the Semi-Arid Tropics

IFPRI – International Food Policy Research Institute

IITA – International Institute of Tropical Agriculture

ILRI – International Livestock Research Institute

IPGRI – International Plant Genetic Resources Institute

IRRI – International RICE Research Institute

IWMI – International Water Management Institute

World Agroforestry Centre (ICRAF)

World Fish Venter

Annex 2

The list of plant genotypes used for transferability assessment and phylogenetic analysis

Annex 2
List of the accessions used for transferability assessment and phylogenetic analyses

Species	Sub-species	Genome	Accession	Country	Code
			A 4	AFG	TAE-A4
			AIFENG NO 4	CHN	TAE-AIFENG
			APACHE	FRA	TAE-APACHE
			AURORE	AUS	TAE-AUTORE
			BALKAN	YUG	TAE-BALKAN
			CHINESE SPRING	CHN	TAE-CHINESE
			CHORTANDINKA	RUS	TAE-CHORTA
			CHYAMTANG	NPL	TAE-CHYAMT
			COPPADRA	TUR	TAE-COPPAD
			COTIPORA	BRA	TAE-COTIPO
			GLEANLEA	CAN	TAE-GLENLE
	aestivum	AABBDD	GOBOLLOI 15	HUN	TAE-GOBOLL
			HOPEA	FIN	TAE-HOPEA
			M708/G25/N163	ISR	TAE-M708/G
			MISKAAGANI	LBN	TAE-MISKAA
			MOCHO DE ESPIGA BRANCA	PRT	TAE-MOCHO
			NP 120	IND	TAE-NP120
			NYU DAY	JPN	TAE-NYUDAY
			OPAL	DEU	TAE-OPAL
			ORNICAR	FRA	TAE-ORNICA
			PITIC 62	MEX	TAE-PITIC
			SEUSEUN 27	KOR	TAE-SEUSEU
			ZANDA	BEL	TAE-ZANDA
			EPEAUTRE BLANC	FRA	TSP-BLANC
			EPEAUTRE BLOND OU DORE	FRA	TSP-BLOND
T. aestivum	*spelta*	AABBDD	EPEAUTRE DE L'AVEYRON	FRA	TSP-AVEYRO
			EPEAUTRE NAIN	BEL	TSP-NAIN
			EPEAUTRE NOIR VELU	FRA	TSP-NOIR
			CANUTO	GBR	TCO-CANUTO
			MIDEA	ITA	TCO-MIDEA
	compactum	AABBDD	RYMAR	USA	TCO-RYMAR
			KUBBWEISEN	SWE	TCO-KUBBWE
			LO TO MAI	CHN	TCO-LOTOMA
			TSPHAEROCOCCUM	FRA	TSH-SPHAER
			LANDRACE 1	IND	TSH-LAND01
	sphaerococcum	AABBDD	LANDRACE 2	IND	TSH-LAND02
			LANDRACE 3	PAK	TSH-LAND03
			81128	AFG	TSH-81128
			TMACHA	FRA	TMA-MACHA
			LANDRACE 4	GEO	TMA-LAND04
	macha	AABBDD	LANDRACE 5	GEO	TMA-LAND05

			LANDRACE 6	GEO	TMA-LAND06
			102 V	SUN	TMA-102V
			LANDRACE 7	ARM	TVA-LAND07
			LANDRACE 8	ARM	TVA-LAND08
	vavilovi	AABBDD	247 V	?	TVA-247V
			479 V	?	TVA-479V
			7110 BV	?	TVA-7110BV
			MAIK	CHN	TPE-MAIK
	petropavlovskyi	AABBDD	LANDRACE 9	CHN	TPE-LAND09
			LANDRACE 10	CHN	TPE-LAND10
			94750	GEO	TCA-94750
			94753	GEO	TCA-94753
	carthlicum	AABB	94755	GEO	TCA-94755
			251914	TUR	TCA-251914
			532479	GEO	TCA-532479
			45239	ITA	TDM-45239
			45351	IRA	TDM-45351
	dicoccum	AABB	45354	RUS	TDM-45354
			45383	BUR	TDM-45383
			352365	GER	TDM-352365
			45963	JOR	TDS-45963
			46253	TUR	TDS-46253
	dicoccoides	AABB	46470	SYR	TDS-46470
			113302	IRA	TDS-113302
			467014	ISR	TDS-467014
			82715	TUR	TDU-82715
			82697	TUR	TDU-82697
			82726	TUR	TDU-82726
			84866	SYR	TDU-84866
			95920	SYR	TDU-95920
			AMIDONIER BLANC BARBU	FRA	TDU-AMIDON
			ARTMET 910	FRA	TDU-ARTMET
			B6RTCHIR	BUL	TDU-B6RTCH
			BRUMAIRE	FRA	TDU-BRUMAI
T. turgidum			DURENTAL	FRA	TDU-DURENT
			GLOIRE DE MONTGOLFIE	ALG	TDU-GLOIRE
	durum	AABB	IXOS 9442	FRA	TDU-IXOS
			JAIRAJ	PER	TDU-JAIRAJ
			KUBANKA	RUS	TDU-KUBANK
			LLOYD 945	FRA	TDU-LLOYD
			NEODUR 91	FRA	TDU-NEODUR
			PERU 1	FRA	TDU-PERU
			PETANIELLE BLANCHE	FRA	TDU-PETANI
			POLOGNE BARBU	FRA	TDU-POLOGN
			POULARD BEAUCE BARBU	FRA	TDU-BEAUCE
			POULARD BLEU CONIQUE	FRA	TDU-BLEU
			PRIMADUR	FRA	TDU-PRIMAD

			VILLEMUR	FRA	TDU-VILLEM
			14140	?	TPO-14140
			45274	TUR	TPO-45274
	polonicum	AABB	68405	?	TPO-68405
			330554	CHY	TPO-330554
			384266	ETH	TPO-384266
			7786	ETH	TTU-7786
			134952	POR	TTU-134952
	turgidum	AABB	191104	SPA	TTU-191104
			341300	TUR	TTU-341300
			387456	ETH	TTU-387456
			68191	?	TMO-68191
			68206	?	TMO-68206
	monococcum	AmAm	68212	?	TMO-68212
			74036	?	TMO-74036
			81051	?	TMO-81051
			68182	?	TBE-68182
			68183	?	TBE-68183
T. monococcum	*boeoticum*	AbAb	68184	?	TBE-68184
			68185	?	TBE-68185
			68186	?	TBE-68186
			77089	?	TUR-77089
			77090	?	TUR-77090
	urartu	AuAu	78092	?	TUR-78092
			78096	?	TUR-78096
			78097	?	TUR-78097
			8	?	AESPEL-8
Ae. speltoides		SS	25	?	AESPEL-25
			37	?	AESPEL-37
			38	?	AESPEL-38
Ae. searsii		SsSs	3	?	AESEAR-3
			4	?	AESEAR-4
			CLAE 70	?	AEBIC-70
Ae. bicornis		SbSb	CLAE 47 1323	?	AEBIC-47
			CLAE PI 330486	?	AEBIC-330
Ae. longissima		SlSl	1	?	AELON-1
			PI 604110 AEG-280-37	?	AELON-604
			13	?	AETAU-13
			15	?	AETAU-15
Ae. tauschii		DD	32	?	AETAU-32
			33	?	AETAU-33
			42	?	AETAU-42
Ae. umbellulata		UU	CLAE 6614 A	?	AEUMB-6614
			PI 222762 1823	?	AEUMB-222
Ae. peregrina		UUSvSv	PI 487202 SY20160	?	APE-487202
			PI 487278 SY20240	?	APE-487278
			PI 54217684 TK 157008	?	ACO-542176

Ae. comosa subven	MM	PI 551039 JM 3738	?	ACO-551039
		PI 551047 JM 3856	?	ACO-551047
Ae. ventricosa	DDMvMv	10	?	AEVEN-10
		11	?	AEVEN-11
		17	?	AEVEN-17
Hordeum vulgare	HH	ALEXIS	?	HVU-ALEXIS
		CFL 86 126	?	HVU-CFL86
		MOREX	?	HVU-MOREX
		PLAISANT	?	HVU-PLAISA
		STEPTOE	?	HVU-STEPTO
Secale cereale	RR	APART	?	SCE-APART
		DANKOWSKYNOWE	?	SCE-DANKOW
		MERKATOR	?	SCE-MERKAT
		PROTECTOR	?	SCE-PROTEC
		SCW 3	?	SCE-SCW3
Tritical		Ampiac	FRA	TRITI-AMPI
		Bienvenu	FRA	TRITI-BIEN
		Matinal	FRA	TRITI-MATI
		Tricolor	FRA	TRITI-TRIC
		Lamberto	POL	TRITI-LAMB
		Zeus	FRA	TRITI-ZEUS
		Trimaran	FRA	TRITI-MATRA
		Carnac	FRA	TRITI-CAMA
Lolium perenne		Clerpin	FRA	LOLIUM-CLE
		Clerpin Pature	FRA	LOLIUM-PAT
		Herbie	NL	LOLIUM-HER
		Kerval	FRA	LOLIUM-KER
		Magella	NL	LOLIUM-MAG
		Ohio	NL	LOLIUM-OHI
Avena sativa		Kassandra	GRC	AVENA-KASS
		Pol	SWE	AVENA-POL
		Revisor	DEU	AVENA-REVI
		Rhea	FRA	AVENA-RHEA
		Tomba	DEU	AVENA-TOMB
Zea mays		Pa91	?	ZEA-PA91
		B73	?	ZEA-B73
		F2	?	ZEA-F2
		Mo4	?	ZEA-MO4
		A188	?	ZEA-A188
Oryza sativa		AZUCENA	?	ORYZA-AZU
		KASACATM	?	ORYZA-KASA
		NIPPONBARE	?	ORYZA-NIPP
		KONL	?	ORYZA-KONL
		IR64	?	ORYZA-IR64
Brachypodium	*sylvaticum*		?	BRACH-SYLV
	distachyum		?	BRACH-DIST

Annex 3

List of EST-SSRs developed from common wheat

Annex 3

List of EST-SSRs developed from common wheat

Marker	Forward primer(G) 5' → 3'	Reverser primer (D) 5' → 3'	GC%
cfe1	CGTGACGAGCATGAGCAC	GACAGGAGGGGGAAGAAATC	57,89%
cfe2	CTTCGCCGACAAGAAGAAGT	CGGCACGTACTCCACCTC	57,89%
cfe3	TGCTACTTTTGGTTACCGGC	GTCAGGTGTTTCCACGCC	55,26%
cfe4	AAGCGGACGTAGCAGGTG	CGACCATGACTCCCCAAG	61,11%
cfe5	TTTCTCGCCAGTATGTATGGG	AAACCCTAGCCACCCTCG	53,84%
cfe6	CTTCCCAAATGGCGACAC	ACAGCAAGAGGAACCACACC	55,26%
cfe7	TCCGACCGCTTCCACTAC	TGTCATAGTTTTCAGCCCACC	53,84%
cfe8	ACGATAAGATGGAAGGCGTG	CTGCACCTCACCAAATCAGA	50%
cfe9	TTCCTTCCAGTATCGTTGGC	AGGACTGCGGGTTGATTTC	51,28%
cfe10	TCGCGTAGTCCATGCAGTC	ATGGCTATCTATAAACACCGGC	51,21%
cfe11	GGAATCCTTGCCCTGGTC	GCTCTCCACGGTTCGTTC	61,11%
cfe12	TTTCGCTTGTCGAACGGT	TGCACTTGTTAGAGGAGAATCC	47,5%
cfe13	AAATCCAAGATGTGCCAAGG	TCGCCGCCAACTACTACC	52,63%
cfe14	CGTCCTTGGGCTGGTAGTAG	CCGCAGAGCACTCCACTT	60,52%
cfe15	AAGTCCTCTCCAGCATCGG	ATTTGGCAGAATCAAATCCG	48,71%
cfe16	GAAACACTGACAGCAGGAAGG	AGAAGGCGAGGACGATGAC	55%
cfe17	CGCCATCTTCACCATCATC	ATCAAATCCGAGACTGTGCC	51,28%
cfe18	CAGGGGATATAAGAAGGAGCG	CGAGACGACGAACGAGGT	56,41%
cfe19	AAGGTGTCGCCGTAGTTCAC	CAGTTGGAGCAGCAGGTGTA	55%
cfe20	GGTTGGGAGACCATTGAGAA	GGACGCGAAGATCCATTG	52,63%
cfe21	GCCAGACACAATCCCAGG	ATCGGAATCATAGTGGTCGC	55,26%
cfe22	AGGACGTGAAGATCCATTGC	GGTGGCTGGGAGACTATTGA	52,5%
cfe23	TGCGTCACCACCTTCTACC	GAAGACTAACCAGAGCAGGCA	55%
cfe24	AAAAGCCGAATCATGGACAC	AGCTGGTACTTGCGGACG	52,63%
cfe25	GGGCTCTCCACTCTCTTGC	TCCGTTCCTATGGGTGACAT	56,41%
cfe26	ATGACCCTAGAAGGCGGTG	ATGCTCAAGCCGAGGAAGTA	53,84%
cfe27	GTTGAACATGATGCTGGGTG	TCAAATCCAATGAGTCAAGCC	46,34%
cfe28	ACTATTCACTCCGCCCTCCT	GCATTCTTTCCAGAGCAACC	52,5%
cfe29	CAGACTCCAGAGAGCGCC	GACGAGGTTGTGGTTCGC	63,88%
cfe30	GATCGAGGAGTGAGTGAGGC	TATACAAATGGCTTGCAGTACG	50%
cfe31	TGGATTTCCTCCATCTCCAC	CGATGATGGTCACGTTTTGA	47,5%
cfe32	AGATCCTCTCCACCATCGC	CCACACTGAGCATTCCTGC	57,89%
cfe33	CAACATCCTGGGCATCAAC	CATGCGAGCTGGTGTACG	56,75%
cfe34	ACAGCAGGCATCCACTATACG	GCCTAGTTCGATGACAAGCA	51,21%
cfe35	GCACACCCCCAGCTTCAG	GAGGCGGATCAGGAGGAG	66,66%
cfe36	GATCTGGAGGAGGCGGTAG	CTTCAGCGTGTCGATGGG	62,16%
cfe37	TCCTCGTCAACTACTGCGG	GGATGGATTGTAGATCATGCG	52,5%
cfe38	GCTCTTCTTCACCTTCGCC	CATTGAGGTTGATGCCCAG	55,26%
cfe39	GTGGAAATGGAGGACGCTTA	AGCTTCAAGGGGGCTGTC	55,26%
cfe40	CTGCCCCTTTTCTCCGTC	TCGCGTGATGATGCTGAT	55,55%
cfe41	CCGGAGAAGAGCACCTAGC	TACGAAAACCTGACCGCC	59,45%
cfe42	CAGCTTCTCCTCCTCGGAC	AGAAGGGAGTGTCGATGGTG	58,97%
cfe43	AGAAAGGGGTGTCGATGATG	AGCAGACGATGTGGTACGC	53,84%
cfe44	AGCTGGAGAAGGAGAACAAGG	CACATAGATCGTGTCGCGTT	51,21%
cfe45	GTGCTGGTGCTGGTGATG	GAGTTCACCCCCCGATCC	63,88%
cfe46	TCACAGCATTGAACTGGCTC	TGTAGCACATGAACACCAAGC	48,78%
cfe47	GGCTGCTCTTCTCCTGTGAC	TGCACCGATCAACACCAG	57,89%
cfe48	GAAGAGCCTCCCGGTGTC	GGTTTCATCCATCGTCCG	61,11%
cfe49	CCAGCGCCTCAAACAAAG	CTTGTCCCCGTAGCCTTG	58,33%
cfe50	ACCTTCCCCTTGTAGGTGCT	TCGTCTGCTCTTCCTCCG	57,89%
cfe51	TCTTGCTTCCTCCATCAACC	ATCGCGGAGCCCTTCTAC	55,26%
cfe52	TGTCGTAGAAGGGCTCCG	AAACCCTACCTCCTAGCTCCC	58,97%
cfe53	TGGACCGCAGAGACTTCG	GTCCGCCCAAACCCTACC	63,88%
cfe54	GAAGCCCATGTCATCATCG	CCTGGAGAAGAGGAGCGTC	57,89%
cfe55	GGAGATCGTCGTCATCCG	TCCTTGCTGTGGAGGGTAAC	57,89%
cfe56	TGCTATCCTCGTCGCCTC	GTGGCCTGGGTAGCCTCC	66,66%
cfe57	TGAACAAACTCAGGCCAAGA	CACCTCCTCCACCGTGTC	55,26%
cfe58	CTGATCCGTCCGTCCATTC	TCCATAGGAACCGGATATACAA	48,78%

cfe59	CAGTGGGTGTACTGGGTCG	CATGGTGATGGCAAGGAAG	57,89%
cfe60	ACCTCTCTCCACTCCGGC	CCACCTCGTACACCTCACG	64,86%
cfe61	CTCCCCAAATTCCACCCTAT	GACCGGGAACCTGTCCTC	57,89%
cfe62	GAGCGAGATCCAGTCCAGAG	AACCAGCCTTGACCATTCC	56,41%
cfe63	AAGAGGTCGGGGAACAGG	GTGATGAGGAGCTTGGTGC	59,45%
cfe64	CACTTACCAAACTCCGGCTC	ACGCCTTCGACACCGAAT	55,26%
cfe65	TGTCCTCACACTCGCTCATC	CACCTTGTAGCCGATGACG	56,41%
cfe66	ACTGGATCTTCTGGGTCGG	ACAGAGAGGACACTGAACGACA	53,65%
cfe67	CATCCATCCATCCATCAGAG	ATGGCCCGGTAGAAGGAC	55,26%
cfe68	CGCCGTCATCTACAACAAGA	CATGCACAGACACTGAACGA	50%
cfe69	TGCAACTTCTCCTCCTTCGT	CGCTGGTTTTAGAGGACTGG	52,5%
cfe70	TTACTCACCGCGCTCCTC	CGTCTTGGTCAGGAACGG	61,11%
cfe71	ACGACACCGCGACTTCTC	GCATGTGCCCTCTCCATC	61,11%
cfe72	GACGGCCTCGATCTCCTT	CACCTCACCTCATCTCACCA	57,89%
cfe73	ATCCCACTCGATCATCTTGC	GAGTCCTTCACCCGCCAC	57,89%
cfe74	AGGCCGTAGGGACCATTC	TCTAAAAGCACCGGACGC	58,33%
cfe75	GGGAATACGTGCGTGAGATT	GAACCGGAGGAGTCCGTG	57,89%
cfe76	CCGGTCTAGTCTCAAGTGTGG	TTGAAGAGCAGCGACTTGC	55%
cfe77	AGCTCTCGGCTCACCTCTC	CAGTGTCACCGGCTCGTC	64,86%
cfe78	TGGATCACGAAGTTGTGGAA	ATCGAGGAGGAGGGGCTC	55,26%
cfe79	CTCCTTCATCCAGGGGCT	AGAACCACTCCAAGACGGC	59,45%
cfe80	TTCAGACTGGCCTCCGTC	GTACAGCATCGGCAAGGAC	59,45%
cfe81	CGTCCGATCAACTCGACC	ACGCTTGCACCTCAGTCC	61,11%
cfe82	AGGCTGAGCGTGAACACC	CAAGAGCACGACGAATTTAGC	53,84%
cfe83	CCTCGATGAAGAACCCGTC	TTTCGGAGTTCCCACGTC	56,75%
cfe84	CAACCTCCGACGAGGAGTC	GAGCGACGGGTTCTTCATC	60,52%
cfe85	ACCTTGAAGCAACAGCCG	CCGTAGGGTTTTGGGATTTT	50%
cfe86	CGTCAGTTCAAATGGCTGC	TCCAGGAATGGGTTTACTGC	51,28%
cfe87	GAGGTTCTTGTGGAGCATCG	CAACTCCTTCGACACCTTCC	55%
cfe88	GCTTACACAGAAGGGCGATT	GGTCCTGGTTGTCCGTTG	55,26%
cfe89	TGGGAATAACACATAGCAGTGG	TGCTTTTCAGTCAGTCACCG	47,61%
cfe90	TGCTCCCTCATGTACTGGC	CAGAAGACGCACACACGC	59,45%
cfe91	CAAGCGAAACCAACGGAG	CCACTACTCAAGAGGCGGAG	57,89%
cfe92	CAGCCAGCCTTTCTTCTCC	TTGGGGACCAACTGGAATAA	51,28%
cfe93	CTAGGGTTAGGTCAGTTTCCCC	GCTCTCCACCTGCGTGAT	57,5%
cfe94	ATGACCTTCACCCGCAAG	TATCGCGCTGTTGATTTCC	51,35%
cfe95	GAACCATGAACCAGATTTGACA	ATACATTCGAGCCACCCTTG	45,23%
cfe96	CATTGGTCTGTTTGACAACTGC	ATCCTGATTCGGACTTTCCC	47,61%
cfe97	GCTTCAATCAAGGACAGGATG	TCCATTTGGGTAACACGGAT	46,34%
cfe98	AACAACCGCTGAGATCGG	GATTCATGGCGAGGTCGG	58,33%
cfe99	CAGGCAGACACGCAACAG	CACAGTCAACTCCTTTGTACGC	55%
cfe100	GCAGTTCTTCCAGTTCAGAGG	AGGGACAACAGTTACGTGGC	53,65%
cfe101	GAACATGCAAGACACGAGTAGC	TACTTCAGCCAGGGCCAG	55%
cfe102	AGAACAGGAGCACGAGCAG	GACGGAGGAGGGTACTTCG	60,52%
cfe103	TCGCACCAGCTACAACACTC	CCATGATTTGTCCGCTTCTT	50%
cfe104	TTGCAGCCTAGCGAGCAG	CCGGTGTGCTGTCAGATGT	59,45%
cfe105	GATGCCGTGGAGCAGTCT	GATGAGCCACATGAATGCC	56,75%
cfe106	CGTCGATCCTATACTACGGAGG	GGTACAAGCCCTGCTCGAC	58,53%
cfe107	TAGATCCGTTTAGCGCCG	GGCACACGGGTACAATCTCT	55,26%
cfe108	AGCGCCAATACTTATCCCCT	GTCCTCTAAGTCCATCAGCCC	53,65%
cfe109	CTCATCGAAGGGGTTGCG	ACAGGCGAGGGAGAAACC	61,11%
cfe110	AGAGCCGAAATAGTCTCGCA	TGCCACCATCCTGAGCTAC	53,84%
cfe111	TACGGCTCCAAGAACGAGAT	CTGCCCACTGCATGTCTG	55,26%
cfe112	GACTACATGCGGGCCATC	GACAGGAGCAGCATCGACC	62,16%
cfe113	CAGAAGTGAGAATGAATGAGCG	AACACAGCCACAGCACTGAC	50%
cfe114	AGGGCAAGAGCATGACG	ACCAAAGCGTTCACCGATAC	54,05%
cfe115	GATGACCAAGAGACCAGGGA	ACATACAATGCTTCCGCACA	50%
cfe116	CGAGTCCTTCACCTCGGTC	CTGCCCTTGCTCACCTTG	62,16%
cfe117	ATATCGCTCGCGCATCTAAT	AGATTCCTCTGAACTAGCCCG	48,78%
cfe118	GAACGCCTCCATCAGCTC	ATCCGCATTCATCTCCTTTG	52,63%
cfe119	CAAACGCCAACCTCCTCC	GGTGTTGAGCACACGCAG	61,11%
cfe120	CACGTCCTGCTTGTGGTAGA	CGAGACACACGCTGGTTG	57,89%
cfe121	TGTGCTGGAACTCCTCACC	AACGCACCTCCTCCCTTC	59,45%
cfe122	GCGATCTTGCAGGTCATGTA	AGAGAGGGCAAAGTAAGCTCG	51,21%

cfe123	CTCGTCAGGAAACCGTCG	CGTCGAAGCAACACAATTACA	51,28%
cfe124	TCTCCAGCAGCAATAACGG	TTACAGAGTCGCAAACGGC	52,63%
cfe125	AGGACTTGGCCCTAACGC	AACGATGGAACAAGGAAACG	52,63%
cfe126	GCAGGCCAACACATCCTTAT	AGACCCTCCTGACCGACC	57,89%
cfe127	CGAGTACAGCCCCAGCCT	GACCAAGGACAACCGCAC	63,88%
cfe128	GGCATCCATGTCCTTCTCTC	TCTGTCTCCTTAGCTTTCCCC	53,65%
cfe129	TAGCGATTTGTTGGTTGTTCG	ACGTATCTACAAGCACACGGG	47,61%
cfe130	GACCCAAAACCAACACCG	CGATGGTAGATCGGAGGC	58,33%
cfe131	CGTCTGGGGGTGAGATAATG	ATGCTTCAGGTGTGGCTTCA	52,5%
cfe132	ATATGTCGAGCTTCGGCG	GGATTGAAACTGGCAAAGGA	50%
cfe134	CGGGTGATGGAGGAGAGG	TTCCATTCTACTCAACGCAAA	51,28%
cfe135	TAGGAGGGTAAACAAGGAGCC	TGGATTGGAGGAAGGAACTG	51,21%
cfe136	TGGAGACAGAGCCCACAAG	CCTCGATCTGCATGGTCC	59,45%
cfe137	GAGAAATAAGCGGTGCTTGC	GGAACTTCTTGGCGAGATCA	50%
cfe138	CATCGTGCTGCGTCAAAG	CATTATCTTCAGCATAGCCGC	51,28%
cfe139	AGAAGAAGGTGAGAGATGGAGG	CAGGGGAGGTAGAACTCGG	56,09%
cfe140	CCAGTGGCACCTCAACCTC	CTCAAGCTGTAGTCGTTGTTGC	56,09%
cfe141	CACCGTCAAACCAACAACAG	AGGAACTACGCCGTGCTG	55,26%
cfe142	GTACCTCCTGTCAAAGCCCA	GTTCGACGACATCGGAAACT	52,5%
cfe143	CGACTAACGACCAAAGCACA	CATCCACACCCACAAGGAG	53,84%
cfe144	AGCATTGCCCAAGAGCAC	CGTGGGAGAGAACACACGC	59,45%
cfe145	TTGGAGGGGAAGGAGGAC	CAGAGAGAACACACGCAAGG	57,89%
cfe146	CCGAGGCTCTTCACATCG	TCAGAACGACCAAGCAGATG	55,26%
cfe147	GCGTCCGAGGCTCTTCAC	ACTGTCCAGGCGATGCTC	63,88%
cfe148	CGGGCTTTATTGAAACCTCA	GTGTCACGTTGGGCATTAGA	47,5%
cfe149	CTGATTACGCGAGCCCAG	CGCAGAAAGGGCAGTAAGAC	57,89%
cfe150	ACGCACGCACCTGTCTATC	AAGAGCAACCCTGGTAACACA	52,5%
cfe151	CAGATGGGCACTCCCTTG	ACCGTGATGGAGATCGAGAC	57,89%
cfe152	CTTCTTCCGCGACTACCTCA	GCATGTCCTGGTCGTCCTC	58,97%
cfe153	GGTGTGTCCTTCCCATGC	TAATGTCAGCGGAAGAGCCT	55,26%
cfe154	AGAACTTGAACTCTGGGAGCC	TCAACCTCCTCATCGGATTC	51,21%
cfe155	GGCACGAGCTTCCTTCTAGG	GGCTTGACCGTCGTGTACTT	57,5%
cfe156	TGTGCGCCATCTGCTACTC	CTCCTAGATCCCGCGTCTC	60,52%
cfe157	ACGAACTCGGAAGTCTAGCG	CATGATCGCCTCCACCTC	57,89%
cfe158	CGTGGTAGTGTCCGTGACC	CACATGGGCTATGGAGGC	62,16%
cfe159	GAGGATGATGGACGTGCTG	GAGGTCGGCGTAGCTCTG	62,16%
cfe160	TTCCCTTCCCCCTCACTC	TCGACATGGAGAGCATCG	58,33%
cfe161	GTCTGAATATGGGGAGGAGATG	AAAACACCGGCAAGAATCAG	47,61%
cfe162	GCTATTCTGCCCGTTCCTC	ACCATTACCAGGTGAACCCA	53,84%
cfe163	CTTCCCGTCCCCTCTCTC	GCTTGCCCTTGAACTTGC	61,11%
cfe164	GGAACCCAGGCGAGGTAG	AGCCTAGCAGCATAGCATCC	60,52%
cfe165	TCCTAATCCCCCGACCTC	AAACAATTTCTGCTCCCACG	52,63%
cfe166	ACCAGCTCAACAAAATGCG	CACGTTCCACGCTACTACCA	51,28%
cfe167	GCCTTCTTCACCTCGCTG	ACCGCATCTTGTTGAGCTG	56,75%
cfe168	ATGCTCTGAAGTCTAAGGTCGC	CAGAAACCAGAAACAAGCAGC	48,83%
cfe169	AGGAAGAGGAAGGTGAGATGG	GGAACTTCTCCAGCGACG	56,41%
cfe170	ATGAAGTTCGTCGCCGTC	TAGCAGAAGAGGTAACCGAAGG	52,5%
cfe171	AAGATCCGGTACGAGGTGC	ACGTGGTGAGGTTTCTGCTC	56,41%
cfe172	GCCATGCAGAACTGGCTC	TGCCATCCATCGACACTAAC	55,26%
cfe173	GCTGGAGTAGAGCGTGCG	TCGTCCTTCTCCTCGGTG	63,88%
cfe174	CCTCTCCATTCCGTGTGG	AGAAGGGGGACTCGATGG	61,11%
cfe175	GTCCTTGGCCGTCTCCTT	AAGCCTCTCCCAAACCCTC	59,45%
cfe176	GATTGTGCTACGACGGTGC	TCCTTGTCGAGATTCCGC	56,75%
cfe177	GTCGATCCGTGGTGATCTG	CGCCCAACTAGGTCGAATC	57,89%
cfe178	AGCTTGCTATCCATGTCCTCA	GTGCAAGGAGCACTGCGA	53,84%
cfe179	CCAAACACAGTGCAAGCG	TGCTGCCTATCGTACTCGTG	55,26%
cfe180	GCCTGTTCAAAATGCAGGAT	AGTGCTGGGAGGTAATGGTG	50%
cfe181	AAGCCACTCTTTAATCCAGCC	GTGTAGAAGGTGAGCAGGGC	53,65%
cfe182	CTGACGCAGAAGAAGCACTG	TTGCCGTAGAAGGGGTAGG	56,41%
cfe183	TCCGGTGAGAGCATAGCAG	TCCGACCGTGTCTACAAGC	57,89%
cfe184	GCGTCCGCTCACGAGACT	ACCTGTGGCTCCGTTGTG	63,88%
cfe185	AAGCGCGACCAGAAGTACAG	CAGCAACTACGAAACAAAGTGC	50%
cfe186	CAACTGCGACGGACCCTT	GCTGGCTAGTGGTGATACGG	60,52%
cfe187	GCTGACCACCACAGAGACAG	CGGGCTGAAGATGACCAG	60,52%

cfe188	ATCTTCGTAGCATTGGCAGG	GCAAAGAGCTTCTGGAATGG	50%
cfe189	ATACAGAACCGGACACGAGG	CACCAAGGACAACACCACC	56,41%
cfe190	GAGGCTGCCAAGCAGAAG	AGCAAATCAAGCGCGAAC	55,55%
cfe191	GAGGGGCGTGCTTAGCCT	TCTGCAAGGACCCCGAGT	63,88%
cfe192	AGAAGAAGCCCAAGGAGGAG	TCTTTCCCTTCACAACCACC	52,5%
cfe193	TTCAAAGGTGTGAGCGTGAG	ACACTCTTGTCGCCCCAC	55,26%
cfe194	TCGAGAGCCGGAAACCTC	AATTCGGCACGAGCACAG	58,33%
cfe195	CCACAAGTAGATGGATCACCG	GCTGGAGAAATACCCTGCAA	51,21%
cfe196	AATCCATACAACGGTGCCAT	AACTGGAAAGAAGCGCAGAA	45%
cfe197	GCCCAACTTCACCATTTCAT	GTGTGGAGACCATGCGAAC	51,28%
cfe198	CGTCCGGTAACACCAACAG	ACGTCCCGGCAGTAGAAGTA	56,41%
cfe199	ATCATCGTCTGTGGTTTCGC	CGGGACATTGAAACATCAAG	47,5%
cfe200	CAGCACTGGAAATAGCTCAGG	GCGCAAGCAGAACAAGAAG	52,5%
cfe201	CCAGGAACCACCAACGAC	CTTCCTCCCCACTTGTTCC	59,45%
cfe202	CGCGAACTTCCTGGACTC	AAACGAGAAAGGCTCACCGT	55,26%
cfe203	TTCAGAGACAAGAGAATGGCG	TTGCACTTCCAGTTGCCTC	50%
cfe204	CAGAACCAGTGGGAGCAGTT	CGTGTGACGTGTGCCTTC	57,89%
cfe205	TTACCATCGCTCCAGACTCC	GACCTACATCTTCAAGCACGC	53,65%
cfe206	ACGAATTGGACATCGAGAGG	CATCATCCTTGGTGTTCGC	51,28%
cfe207	CCAACATCGTACTCCGGC	CCGTAGTCAATCACCACACG	57,89%
cfe208	CCGACCAGAAAGACGAGAAG	TGGATGGTTGATGACATGCT	50%
cfe209	GAGTCAGGCGATGTACGATG	GCACTACCAACACAGCCAGA	55%
cfe210	TGCAGGTCCTTGGAGTCG	GTCGGAGAGAGGCGATGG	63,88%
cfe211	CCAATGACAAGAAGGGTGCT	GCTCCTTGGACTGGAGGTC	56,41%
cfe212	CCCATGACGACCTTGCTATT	GAAAACCTCGCAGGCAAC	52,63%
cfe213	AGAGGATGGTGTGGCCTG	TCGTGAACGTGGTGATGC	58,33%
cfe214	CGATACCACCTACCCCGTC	GACCTCCATCTCGTCCCG	64,86%
cfe215	TGGCATCTGTAGAGGAGTTGG	GGAGTCCATGACCTTGTCGT	53,65%
cfe216	CTCCTCTGAGCAAATGGTCC	GGATAGGTAGTGGTTCTCGGC	56,09%
cfe217	TTCTCCAACCATACGATCAGG	ACACCGGCGGATATGGAG	53,84%
cfe218	GGATCGAGAGCGAGGACAT	CTCCACTCCCGTCAGCTC	62,16%
cfe219	AAGCTCACCCTCATCGCC	AGGAACAGATGCGCCAAG	58,33%
cfe220	GCCATTGCTGCTGAGCTT	ATATGTCGCCTCGGAATGAG	52,63%
cfe221	CTACTGGAGGTACGCCGAC	TCCAGCCATCTCTTGTCCTC	58,97%
cfe222	CCGACGATGATGTCAATAACC	CAAACCTCTTCCTCTCACGC	51,21%
cfe223	CGCGTCCGTTCTCTTCATTA	GCTTGGCTGCTCTCTGTAGG	55%
cfe224	TCTCCGACAAGCTCTGCTG	GTACGGCACCTCCACGAC	62,16%
cfe225	GCACGTCCTTGCACACTG	CTCCATCCTCATGGCGAC	61,11%
cfe226	CTCGCTCTCGCCTACTCG	TCAGAATAATGGTGTGCCTCC	56,41%
cfe227	CTCCTTGAACTGCACGATGA	GCTCACCAAGAAACTACGCC	52,5%
cfe228	CACACCACCACTGTCCTCC	GAACTCGTGCATCCCGTC	62,16%
cfe229	TCACAGGGATGACGACGAT	GAGCGACGAGGAGCTGAG	59,45%
cfe230	CACGAGAGCTAGGGGAACTG	TTGTGAGAGATATGCCAAGCC	53,65%
cfe231	GGCGACTGCTCTTCTCGG	CAGAAGGGAAAGTTTGCAGC	57,89%
cfe232	TTCTGGTTGTGAGCGAGTTG	ATTGACGGCGAGGTGAAG	52,63%
cfe233	GAGGAAGGGAAGGGAGGAG	GTTGCCGTGGAGGTTGTC	62,16%
cfe234	CCTCCTGCTGCTAATCTTGG	TCTTGGAGATGATGTCGGC	53,84%
cfe235	CGAGGACGACTCTTCTCCAC	TGCAGGGTAGCAGCGATAG	58,97%
cfe236	TAGAGTTCGACGCAGGGC	GCAACTGATGAGCACACGAT	55,26%
cfe237	GAATGCGTGATCTTGGTGG	GCGCCATAGCCTCCTCTAC	57,89%
cfe238	CGATGACAGCGAATGGTG	GCGGCAATGTCCTTCAAC	55,55%
cfe239	GAAGAGGCTGCGGTACAAAG	AATCTTGGAATCGCCGAAC	51,28%
cfe240	GAGGGAGTCGAAGCGGTC	GACGTGGAGTTCGTGGACTA	60,52%
cfe241	GAAGCTGGTCACGTTTACAGG	TTAATCAAGCCACACGCCTT	48,78%
cfe242	GAGTTCCTGCCGAGGTTG	CTCTCCTCTCCCACACAAGC	60,52%
cfe243	CCTCGCAAAGCATAGGTAGC	CTAAAGTTCCTCGGCACGAC	55%
cfe244	CGTCAGGTCAGCATCAACAG	GGTCGAGTCGTATGGCAGC	58,97%
cfe245	GGCACCATGTCCCTTAGTGT	AGCTGGCTTTCTTCCTTGGT	52,5%
cfe246	AGAAACCCAGCCCCAGAC	CGCTTCCTGTCATCTCCTTC	57,89%
cfe247	AGCTTGACGAGCCACGAC	AGTGACAGCAACAATGACCG	55,26%
cfe248	CATCCTCTCCACCAAGCG	GACGAGTAGGACACCCCGTA	60,52%
cfe249	CGTTGCACTTGTCGTTGC	ACCATTTCAGAGCGCGAG	55,55%
cfe250	TCATCATCGCCTCCATCTC	CAACCGCAAGAAACCAGG	54,05%
cfe251	TCGTCGATAGCGTCTCCTG	ACAACCGCACAAGCGAAC	56,75%

cfe252	GTACTCGCTGCCGCTCTC	TGTCCTTCGTGCTCCGTC	63,88%
cfe253	CACCCAGGTCAAGGAGGTC	TCCATCAGAGACAGCAGACG	58,97%
cfe254	CGCTCCTCTTGCTTCCAG	ACTGACCCCCACCTGCTAC	62,16%
cfe255	TCGTACCTGTCATGCCTGC	GATGGAGGTGATGGTGGC	59,45%
cfe256	GGGCATTCACTGGGAAGTTA	CTGCTTTCAGGGTTAGCTGC	52,5%
cfe257	AGCAGGAGGATCAGAAAGAGG	TCGACTCCAGGGTGGTAGAC	56,09%
cfe258	TCAGCTAACTCCTCGGCG	AGGGAAGCAATCCATCTCG	56,75%
cfe259	CACCTCTGCTCCCTGCTAAC	GAGCACTACGACGCGCTG	63,15%
cfe260	GAAGCCTCCGCTGCTAAAC	TGGAAACTGATACAAGGCAGC	52,5%
cfe261	AAGCAGCTCCAAACACCAAG	CTCAGTGACAAGGACGACGA	52,5%
cfe262	AGGGACTGCTCGGTCAAC	ACGAGAGGTATTTAGGCGCA	55,26%
cfe263	GAGGAGGTAGAAGCCGCC	CCGCCCAAGATGTACCAG	63,88%
cfe264	GATTCCCATCCCGTCACC	CAGCCTGTCCGAGATGAAG	59,45%
cfe265	GGCTCTGCTCGCATCTTC	CTGCCCTCTGCTACTGGAAC	60,52%
cfe266	GCCCACATCACACATCAAAC	GACATGGACAAGGTGCAGG	53,84%
cfe267	GACGTAGACCTTGACCGCC	CATTCCATCTCGAACCCATT	53,84%
cfe268	AACTTGAGCAGCATCGCC	AAGAGGGAGACCGATGGC	58,33%
cfe269	GCTTCTCTTCCAGCATCCAC	GCCACCAACATCTTCATCG	53,84%
cfe270	CCCGTAGAAATGGTACTGGTG	GGCATGGGCTCGCACTAC	58,97%
cfe271	ATCCACTTGTAGACCCGGC	ACTCTGCTTCCGCCACTG	59,45%
cfe272	GAGCCTCTCGTCTCCCATC	GCACTTCACGCCGATCAC	62,16%
cfe273	AGACCTGGCCTTTCCTCG	AGGACGCAAACGTACTCTCG	57,89%
cfe274	GACACACTTGCCGTGGAAC	GAAGATCACGAGGACGAAGC	56,41%
cfe275	CCTTCTTCGGCTGCTAACC	CATGCGCCTTACTTAGCCTC	56,41%
cfe276	GCCTGGTTGCTTCCTGTC	GCCCTGCTCCACCTTCTC	63,88%
cfe277	TTCGACTACGGCTTCATGC	GATCTGGCGAGGGAACTC	56,75%
cfe278	ATGCAAGCGAGGTTCGTC	GAGTGAGGCTGCTCGACAC	59,45%
cfe279	AGGAGAGGACGAACTTGGC	GCACACCAACAACCAGAGC	57,89%
cfe280	GACAAGAACCGGATGGCTC	GTTGGAGGTGTTGCGACC	59,45%
cfe281	AACAGATTGGTTGGTCACACA	TGGAACCACTTCCTCTACGC	48,78%
cfe282	GCGGTGTATGGTCTGGAGTT	TTGACATCGTACTCGTCATCG	51,21%
cfe283	AAGCGAAGCCGAGGAAACT	GAAGCGGGAGAAGAACTCG	55,26%
cfe284	CCCACTAGCCAGCCACTC	TGCGGTACTATTCCACGACA	57,89%
cfe285	TGGGTTCACAGTCCATAGCA	AGCAAATCACCTGGGCTG	52,63%
cfe286	ACCACCACATCAAGTGAAAGC	AGGACGAGGAGCTGATGCTA	51,21%
cfe287	ATTTCCTCGCCAACCTGTC	CATACCATTACCGACCCGAC	53,84%
cfe288	TCCCTTCGTCCATGCCTC	GTGGTCCGATCCCGTCAC	63,88%
cfe289	TCGGAGGAGATGAATGGC	CCATTTGAAACAGGCTCCAT	50%
cfe290	AGCAGCAGTATCAGTCCGGT	CGTTTCCTACATACACACTGCG	52,38%
cfe291	CTCTTCCGGCCATCATTACT	TTTCCATTGACAACCTTCACC	46,34%
cfe292	GAGACACCCACATTGGCAC	GAGGAGGACGATGCCGAC	62,16%
cfe293	CCAAATCAGTCATCCGCC	CTCGTCCAGGCTCAATGC	58,33%
cfe294	CTCACCACAGCAGGAGCC	CTTCTACCCTCCGCCCAC	66,66%
cfe295	AATCACAGAAGCAGAGAGGAGG	GGACGACGGTGAGAATGC	55%
cfe296	ATCTCGTCATCGACCAAAGG	AAGTCCAGCGTCAGGCTCT	53,84%
cfe297	TCCCTCGCTCGCTATGATT	GAAGTAGGAGCACACCTCGC	56,41%
cfe298	ACACGATCAGCAGAGGCAG	TTGAAGCCGGAGAGGTTG	56,75%
cfe299	TTCCAGAAGCTCGACGCC	AGGTGGTCGGAGAAGATGC	59,45%
cfe300	GCTGCCATTCATCCACATC	GTGTTCCGTCACGTATGGTG	53,84%
cfe301	CACGCCGTCACTACATGC	CAGGGTCTGAATCGGGTAGA	57,89%
cfe302	TCGTCAACGTCGTCCGAG	CACCGAGAAGGTCTCCTTGA	57,89%

Annex 4

Result of amplification, polymorphism and transferability for 301 EST-SSRs

Annex 4

Result of amplification, polymorphism and transferability of the wheat EST-SSRs.

Marker name	Repeated motif	Number of repeats	Expected product length (bp)	Real product size (bp)	Ampli-fication	Quality	Polymorphism				Polymorphism among at least one mapping population.	Amplification in relatives								Chromosomal assignment
							Al-OP	EU-RE	AR-REC	CT-CS		Td AB	Tm A	As B	At D	Sc R	Hv H	B	Os	
cfe1*		4	150	165	+	A					-	-	-	-	+	-	-	-	-	3D
cfe2*	CGC	4	175	271, 270	+	A	+	+		+	+	+	+	+	+	+	+	+	+	6A, 6B, 6D
cfe3	GAG	4	289	143-150	+	C	+		+		+	+	+	+	-	-	-	-	-	2AL, 2D, 5A
cfe4*	GAG	6	217	222, 228, 236	+	A	+				+	+	+	+	+	+	+	+	-	3B, 4B
cfe5	GGA	6	245		-															
cfe6	CGT	7	239	300	+	B	+				+	+	+	+	+	+	+	+	+	2AL, 2B, 2D
cfe7	GGC	4	313		-															
cfe8*	CG	6	277	291	+	B		+	+	+	+	+	-	-	-	-	-	-	-	4BS
cfe9	TA	22	185	197-209	+	C	+		+	+	+	+	-	-	-	-	-	-	-	-
cfe10	AGG	8	324	315, 326, 341	+	B					-	+	+	+	+	+	+	+	+	7A, 7B,7D
cfe11*	CGG	8	224	240	+	A					-	+	+	-	-	+	-	-	-	4A
cfe12	ATC	8+4	247		-															
cfe13*	CGG	5	227	379, 382	+	A					-	+	+	+	+	-	-	-	-	2AL, 2D
cfe14	TC	4	242	255	+	B				+	+	+	-	+	-	-	-	-	-	2B
cfe15*	TCA	4	135	151	+	A					-	+	+	-	-	-	-	-	-	5A
cfe16	CAT	4	221	238	+	B				+	+	+	+	+	+	+	-	-	-	5D
cfe17*	TCA	4	247	265	+	A					-	+	+	+	+	-	-	-	-	-
cfe18*	GTCA	3	257	273-277	+	A	+			+	+	+	+	-	+	+	-	-	-	-
cfe19	TGC	4	266	281, 291	+	B					-	+	+	+	-	-	-	+	-	7A, 7B
cfe20*	AGG	4	186	142, 155	+	A					-	+	+	+	+	+	-	+	-	7D
cfe21	ATG	5	267		-															
cfe22*	TTC	4	141	158, 161	+	A	+		+	+	+	+	+	+	+	+	+	+	+	4A
cfe23	CGA	5	231	235	+	B		+			+	+	+	+	+	+	+	+	+	1B
cfe24	CGC	5	239	333, 244	+	C	+	+			+	+	-	+	+	-	-	-	-	
cfe25*	ATC	4	110	124	+	A	+		+		+	+	+	+	+	+	+	+	+	1B
cfe26*	CAG	8	187	191, 195	+	A			+		+	+	+	+	-	+	-	+	-	1A, 1B
cfe27	CTG	8	252	262-268	+	B	+				+	+	+	+	+	+	+	+	+	5A, 5B, 5D
cfe28	AGC	7	202		-															
cfe29*	GA	7	201	213, 219	+	A					-	+	+	+	+	+	+	+	-	3D
cfe30*	TGTA	8	194	210	+	A					-	+	+	-	-	-	-	-	-	3A
cfe31	GCC	4	246		-															
cfe32*	CCG	5	119	134	+	A					-	+	+	+	+	+	-	-	-	6B
cfe33*	TACG	3	185	201	+	A					-	+	+	+	-	+	-	-	-	-
cfe34*	TG	8	235	224, 257	+	B		+			+	+	+	-	+	+	-	+	-	4BL, 5A, 5B
cfe35	CCG	4	119		+	B	+	+			+	+	+	+	+	+	+	+	+	6A
cfe36	GCG	4	131	143, 147	+	B	+				+	+	+	+	+	-	-	+	+	4D
cfe37*	TACG	3	150	161, 165	+	A					-	+	+	+	+	+	+	+	-	5A, 5B
cfe38	GCG	5+4	271		-															
cfe39	TG	7	259		-															
cfe40	CGC	5	180	364	+	B				+	+	+	+	-	-	-	+	-	-	6A
cfe41	GCGG	5	195	213	+	B	+	+	+	+	+	-	-	+	+	+	-	-	-	1D
cfe42	GGA	6	221	229	+	C	+	+			+	+	+	+	+	-	-	+	-	7D
cfe43	CCT	4	261	272	+	A	+				+	+	+	-	+	+	-	+	+	-
cfe44*	AGC	4	219	234	+	A					-	+	+	+	+	+	-	+	-	-
cfe45*	TGG	6	105	124	+	A	-				-	+	+	+	+	+	+	+	+	-
cfe46	GGC	6	197		-															
cfe47*	GAA	4	132	148, 157	+	A		+	+		+	+	+	+	+	+	+	+	+	1A, 1D
cfe48	TGC	5	259	268	+	B					-	+	-	+	-	+	+	-	-	-
cfe49	GAG	5	151		-															
cfe50	GGC	4	247	237	+	C					-	-	-	-	-	-	-	-	-	-
cfe51	GGC	7	178		-															
cfe52*	TC	16	160	159-182	+	A	+	+			+	+	-	-	-	-	-	-	-	2B
cfe53*	TC	10	104	120	+	A				+	+	+	+	-	-	-	+	-	-	2AL
cfe54	CAG	5	172		-															
cfe55	CTT	7	218	67	+	c					-	+	-	-	-	-	-	-	-	2AL, 2D, 3D
cfe56	CGG	8+4	156	237,275,304	+	B		+		+	+	+	+	+	+	+	+	+	-	-

cfe57	GGC	16	182	114,130,135	+	C					-	+	+	+	+	+	-	-	-	2AL, 2D
cfe58*	TTA	4	202	222	+	A	+	+	+	+	+	+	+	+	+	-	-	+	-	4A, 4D
cfe59	CCGT	3	223	213.236	+	B	+				+	+	+	+	+	+	-	+	+	4A
cfe60*	TCT	4	136	151	+	A					-	+	+	+	+	+	+	+	+	-
cfe61	AGG	8	126	136	+	D					-	+	-	+	+	-	-	-	-	-
cfe62	CAA	5	247	252	+	D					-	+	-	-	-	-	-	-	-	4A, 6A
cfe63*	GGC	4	149	150.28	+	A					-	+	+	+	+	+	+	+	+	7B
cfe64	AGGCG	5	139	155-160	+	A	+				+	+	+	+	+	+	+	-	+	2B, 5A, 7D
cfe65	AG	9	284	294.3	+	B				+	+	+	+	+	+	-	+	-	+	7D
cfe66*	GGC	4	223	239	+	A	+				+	+	+	+	+	+	-	+	-	2D
cfe67	AG	11	175	186, 222	+	A	+	+			+	+	+	+	+	+	+	-	+	2D
cfe68*	GGC	4	260	382	+	A					-	+	+	+	+	+	+	-	+	
cfe69	CGA	4	178		-															
cfe70	GCA	4	251		-															
cfe71	GCC	6	143	245, 251	+	B					-	+	+	+	+	+	-	-	-	3B, 3D
cfe72	CTT	5	235	247, 250, 265	+	B	+	+	+	+	+	+	+	+	+	+	-	+	-	4BL, 4D
cfe73	TCC	4	150		-															
cfe74*	TCGC	5	233	248	+	A					-	-	-	+	+	-	-	-	-	6D
cfe75*	CTC	7+4	135	149	+	A					-	-	+	+	+	+	+	+	-	7B
cfe76*	GGC	4	136	152	+	A					-	+	-	+	+	+	+	+	+	1B
cfe77	GGC	6	146	160	+	B					-	+	+	+	+	-	+	-	-	1A
cfe78*	CGT	4	218	328, 335	+	A						+	+	+	+	+	-	+	-	1D
cfe79	GGA	4	220	233	+	B					+	+	+	+	+	+	+	+	+	-
cfe80	CA	8	198	200-213	+	B	+			+	+	+	+	+	+	+	+	+	-	6A, 6B, 6D
cfe81	GCG	7	201		-															
cfe82*	GCG	4	254	270	+	A	+	+	+		+	-	+	+	+	+	+	+	-	-
cfe83	GA	14	244		-															
cfe84	CCT	4	128		-															
cfe85	GGC	6	290		-															
cfe86*	CAG	5	133	148, 154	+	A	+			+	+	+	+	+	+	+	+	+	+	1B
cfe87*	GAG	6	143	146, 159	+	A	+	+	+	+	+	+	+	+	+	+	+	+	+	6D
cfe88	CCG	6	227	121	+	D					-	-	-	-	-	-	-	-	-	-
cfe89	GAA	6	241	247-257	+	B					-	+	+	+	+	-	-	-	-	4A, 4BL
cfe90*	CTG	6	159	174	+	A					-	+	+	+	+	+	+	+	+	4D
cfe91	CGC	3	297		-															
cfe92	CGC	6	189		-															
cfe93	CGG	6	238		-															
cfe94	CCAT	6	105	119	+	B					-	+	+	+	+	-	-	-	-	6D
cfe95	CAT	7	247	259, 266	+	B	+				+	+	+	-	+	+	-	-	-	6D
cfe96	CAA	10	135	150, 154	+	B					-	+	+	+	+	+	+	-	-	2B
cfe97	AGCC	3	150	380	+	C			+			+	-	+	+	-	-	-	-	-
cfe98	GTC	7	139	148	+	A					-	+	+	+	+	-	+	-	-	-
cfe99	CCGC	3	234		-															
cfe100	TG	6	234	249, 254, 271	+	B	+				+	+	+	+	+	+	+	+	-	7A, 7B, 7D
cfe101	CAG	4	286	135, 175	+	D					-	-	+	+	+	-	+	-	-	-
cfe102	GCG	5	169	181	+	B					-	+	+	+	+	+	+	+	-	7D
cfe103*	GAAG	3	104	119, 255	+	A				+	+	+	+	+	+	+	+	+	+	1A
cfe104	GCC	5	101	115	+	C	+				+	+	+	+	+	+	-	-	-	-
cfe105*	CAT	6	198	350	+	A		+			+	+	+	+	+	+	+	+	+	-
cfe106	TG	6	157		-															
cfe107	CGA	5	157	166, 175	+	B	+		+		+	-	+	+	+	+	-	-	-	3B, 3D
cfe108	CGG	5+5	278		-															
cfe109	CTC	7	138	150	+	D					-	-	-	-	-	-	-	-	-	-
cfe110*	AC	7	363	420-460	+	A					-	-	-	-	-	-	-	-	-	4A, 4BL
cfe111	TCT	8	228	245	+	c					-	-	-	-	+	-	-	-	-	3D
cfe112	GCG	4	134	148	+	B	+	+			+	+	+	+	+	+	+	+	+	-
cfe113	GAG	4	329	124	+	A	-				-	+	+	-	-	-	-	-	-	2
cfe114	AGT	5	174	193	+	B	-				+	+	+	+	+	+	-	-	-	5A
cfe115	CTA	4	171	153, 188	+	C	-				-	+	+	-	-	-	-	-	-	4BL
cfe116	CCT	6	248		-															
cfe117	GAG	4	121	137	+	B					-	+	+	-	+	+	+	-	-	3D
cfe118	GGA	4	308	130, 415	+	B		+			+	+	+	+	+	-	-	-	-	-
cfe119*	GCC	4	130	212, 226	+	A	+	+	+	+	+	+	+	+	+	+	+	+	+	2B, 7B

cfe120	CTG	6	204		-														
cfe121	CGG	9	107	135, 196, 215	+	B		+			+	+	+	+	+	-	-	+	-	5A
cfe122	AGC	4	140	96	+	D					-	+	-	-	-	-	-	-	-	-
cfe123	CTC	4	212	263	+	D					-	-	-	-	-	+	+	-	-	-
cfe124*	GAACCC	3	263	269, 279	+	A		+			+	+	+	+	+	+	+	+	-	6B, 6D
cfe125*	CAG	6	235	254, 260	+	A	+			+	+	+	+	+	-	+	+	-	-	6A, 6B
cfe126*	TCT	10	224	134, 420	+	A	+	+	+	+	+	+	+	+	+	+	+	-	-	2B
cfe127	TGC	8	142	158	+	C					-	-	-	+	+	-	-	+	-	3B
cfe128	TTC	10	124		-															
cfe129	GAT	5	128	146	+	B					-	-	+	-	+	-	-	-	-	3B, 3D
cfe130	CGA	4	168	178	+	B					-	+	+	-	-	-	+	-	-	-
cfe131	AGC	6	137	152	+	B					-	+	+	+	+	+	-	-	-	3A, 3B, 3D
cfe132*	GGC+GGC	4	145	159, 165	+	A					-	+	+	+	+	+	+	+	-	6A
cfe134*	GATCT	4	228	135	+	C					-	+	+	+	+	+	+	+	+	3A
cfe135*	ACAG	4	197	212	+	A				+	+	+	+	+	+	+	+	+	+	7D
cfe136*	AG	7	160	140,145,149	+	A	+		+	+	+	+	+	+	+	+	+	+	-	1B, 1D
cfe137*	AG	20	267	136,143,145	+	A					-	+	+	+	+	+	+	+	-	-
cfe138*	GC	7	234	219.249	+	A					-	+	-	+	+	-	-	-	-	4A, 7D
cfe139*	GGC	6	185	233	+	A	+				+	+	+	+	+	+	+	+	+	-
cfe140	CAG	4+5	117	125,131,137	+	C					-	+	+	+	+	+	+	+	+	2B
cfe141*	ATC	4+5	311	327	+	A					-	-	-	-	-	-	-	-	-	-
cfe142*	GAT	5+5	137	154	+	A	+				+	+	+	-	+	+	-	+	-	4A
cfe143	CAGG	4	150	166	+						-	+	+	+	+	+	+	+	-	5D
cfe144	CCT+ GGC	4+9	159		-															
cfe145	GGC	6+5	248		-															
cfe146	AGA	4	307		-															
cfe147	CGG	4	227	265	+	C					-	+	+	-	-	-	-	-	-	-
cfe148	TATG	4	152	145.169	+	B	+		+		+	+	+	+	+	+	-	-	-	4A, 4D
cfe149	TCC	7	240		-															
cfe150*	CGT	6	118	132	+	A		+			+	+	+	+	+	+	-	+	-	1A, 1B, 1D
cfe151*	CAC	4	150	165	+	A					-	+	+	+	+	+	+	+	+	-
cfe152*	CGA	5	150	263	+	A	+			+	+	+	+	+	+	+	+	+	-	6A, 6B, 7A
cfe153	AAC	4	238		-															
cfe154*	CGC	4	141	158.162	+	A	+			+	+	+	+	+	+	+	+	+	+	3A, 3D
cfe155	CTAGG	5	249		-															
cfe156	GGAC	6	288	286.298	+	C					-	-	+	+	+	+	+	+	+	5D, 6A, 6B
cfe157	GAG	6	323		-															
cfe158*	GCC	4	130	147	+	A					-	+	+	+	+	+	+	-	-	-
cfe159*	CAG	6	163	183.194	+	A					-	+	+	+	+	+	+	+	+	5A, 5D
cfe160	CGC	7	109	123	+	C					-	+	+	+	+	-	-	+	-	4A
cfe161*	CATGG	3	135	153	+	A					-	+	+	-	-	-	-	-	-	3A
cfe162	GGCGGT	3	232	247.252	+	B		+	+		+	+	+	+	+	-	-	-	-	5B, 5D
cfe163	CCG	8	102	111-117	+	B					-	+	+	+	+	+	-	+	-	3A, 3B, 3D
cfe164	GCG	5	134	148	+	B	+			+	+	+	+	+	+	+	+	+	-	6A, 6B, 6D
cfe165	GTGC	4	303		-															
cfe166	CGT	7	131	142.148	+	A	+			+	+	+	+	+	+	+	+	+	-	7A, 7B, 7D
cfe167*	GGC	4	187	202	+	A	+			+	+	+	-	+	+	+	+	-	-	1A
cfe168*	CAG	4	153	171	+	A				+	+	+	+	+	+	+	+	+	+	6A
cfe169*	CGG	4	150	165	+	A					-	+	+	+	+	-	+	+	+	2B, 2D, 6A
cfe170	CGC	4	158	351-383	+	B	+	+			+	+	+	+	+	+	-	+	+	-
cfe171	ATGC	5	310	213	+	A			+		+	+	+	+	+	+	+	+	+	-
cfe172*	TG	8	123	192.211	+	A	+			+	+	+	+	+	+	+	-	-	-	3D
cfe173	CGC	6	131	142	+	C	+			+	+	+	+	-	+	+	-	-	-	3A, 3B, 3D
cfe174	GAC	4	254	249	+	A					-	-	+	+	+	+	-	-	-	-
cfe175*	GGC	6	216	198	+	A					-	+	+	+	+	+	+	+	+	2AL
cfe176	GCG	4	100	126-150	+	D	+			+	+	-	+	+	+	-	-	-	-	-
cfe177	GGA	4	138	417	+	D	+	+	+	+	+	+	+	-	-	-	+	-	-	-
cfe178	GCC	4	120	135	+	D	+				+	-	-	-	-	-	-	-	-	-
cfe179	AGG	5	315	327	+	C	+	+	+		+	+	+	-	+	-	-	-	-	6A
cfe180	GCA	8	118	90,123,156	+	B		+			+	+	+	+	+	+	+	+	-	5A, 5B, 5D
cfe181	TC	8	218	234.249	+	B			+	+	+	+	+	+	+	+	+	+	-	4D
cfe182	AAC	4	238		-															
cfe183	AG	14	215	229-240	+	B	+		+		+	+	+	+	+	+	+	+	+	2B

cfe184	CGG	4	100	115	+	C		+			+	+	+	-	+	+	-	-	-	-
cfe185	TG	10	281	292-294	+	B					-	+	+	+	+	+	-	-	-	1A, 3A, 3B, 3D
cfe186*	TG	7	200	218	+	A		+	+		+	+	+	+	-	-	-	-	-	5A
cfe187*	GGC	4	249	257	+	A	+				+	+	+	+	+	+	+	-	+	-
cfe188*	TGC	5	244	229, 262	+	A	+				+	+	+	+	+	+	+	+	+	4BL
cfe189*	TGG	5	221	211, 239,	+	A		+			+	+	+	+	-	+	-	-	-	1A
cfe190*	CAC	6	266	270, 279	+	A	+				+	+	+	+	+	+	-	+	-	1B,1D
cfe191	CCG	6	132	128-144	+	B	+				+	+	+	+	+	+	+	+	+	1B
cfe192	CGC	6	309	236-	+	D		+		+	+	+	+	+	+	+	+	+	+	-
cfe193*	GGC	4	270	283	+	A	+	+	+	+	+	+	-	-	+	-	-	-	-	1A
cfe194	GT	22	156	177	+	D					-	+	+	+	+	-	-	-	-	-
cfe195*	GAA	4	143	387, 396	+	A	+	+	+		+	+	+	+	+	+	+	+	+	3A
cfe196	GGTT	5	110	118,122,127	+	B					-	+	+	+	+	+	+	+	-	6A, 6B, 6D
cfe197*	TAA	4	293	305.309	+	A					-	+	-	+	+	-	-	-	-	4BL, 4D
cfe198	AGC	6	196	204.212	+	B	+				+	+	+	+	+	+	+	-	-	-
cfe199*	TGC	9	209	218-227	+	A	+				+	+	+	+	+	+	-	-	-	-
cfe200	GCT	4	213	274	+	D														-
cfe201	GGAGG	4	126		-															
cfe202*	CGC	6	262	361	+	A					-	+	+	+	+	-	-	+	-	7A, 7B
cfe203	CAC	4	191	133	+	C				+	+	+	-	-	-	-	-	-	-	-
cfe204	CCTT	4	192	195,201,204	+	B	+			+	+	+	+	+	+	+	-	+	-	4A, 5B, 5D
cfe205*	CAG	5	129	135.144	+	A					-	+	+	+	+	+	-	+	-	4D
cfe206	CGC	5	169		-															
cfe207	GCG	7	139		-															
cfe208*	AGG	4	249	262	+	A	+			+	+	+	+	+	+	+	+	+	+	5A
cfe209	CAG	6	217	228-259	+	B					-	+	+	+	+	+	+	+	-	2B, 2D
cfe210	CCG	5	121	134	+	B					-	+	+	+	+	+	+	+	+	-
cfe211	GGC	5	156	120,158,161	+	B	+				+	+	+	+	+	+	+	+	+	3B, 5B, 5D
cfe212	CGG	7	207	213,217,222	+	B					-	+	+	+	+	+	+	+	+	2B, 2D
cfe213	CGC	7	288	302	+	B	+				+	+	+	+	+	+	-	-	-	4D, 7A, 7B
cfe214	CCG	6	107	122	+	B	+				+	+	+	+	+	+	+	+	-	6B
cfe215	GAG	6	200		-															
cfe216	CCT	6	319		-															
cfe217	CGC	5	246		-															
cfe218	GGGGGC	4	181	193	+	B					-	-	-	+	+	+	-	+	-	4D
cfe219*	CCG	4	208	340	+	A					-	+	+	+	+	+	+	+	-	-
cfe220	CTG	5+4	246	239.262	+	B	+				+	+	+	+	+	-	+	+	-	2AL, 3B, 3A
cfe221	CAG	6	250		-															
cfe222	GAT	4	238	258	+	C				+	+	+	+	+	+	+	-	-	-	6A, 6D
cfe223*	ATAC+TCCT	5+7	157	164-173	+	A		+	+		+	+	+	-	-	+	-	+	+	7B
cfe224	GCC	7+4	275	185-219	+	D	+	+	+	+	+	+	+	+	+	+	+	+	-	-
cfe225*	ACG	5	349	359	+	A					-	+	+	-	+	-	+	+	-	3D
cfe226*	TCTCC	4	170	143	+	D					-	+	+	-	+	-	-	-	+	-
cfe227*	CGC	5	161	177	+	A	+		+		+	+	+	+	+	+	-	+	-	2D
cfe228*	CTG	13	207	214	+	A	+		+	+	+	+	+	-	-	+	+	+	-	4A
cfe229*	ACA	4	298	315	+	A					-	+	+	+	+	+	+	+	-	5B
cfe230	CTC	8	317	334	+	B	+				+	+	+	+	-	-	-	-	-	5B
cfe231	CGG	4	335	360	+	D					-	-	-	-	-	-	-	-	-	-
cfe232	GTG	4	245	131	+	B			+	+	+	+	-	+	-	-	-	-	-	-
cfe233	CGG	4	186	201	+	C	+				+	+	-	+	-	-	-	-	-	-
cfe234*	GTCC	6	237	251-571	+	A					-	+	+	+	+	+	+	+	-	4A, 4D
cfe235	CGC	4	186	145-258	+	D					-	+	+	+	+	+	+	-	+	-
cfe236	CGTA	4	110	120-127	+	B	+	+			+	-	-	-	-	-	+	-	-	2B
cfe237	GGT	5+4	274		-															
cfe238*	GAG	6	141	157	+	A	+			+	+	+	+	+	+	+	+	+	-	-
cfe239	GGT	6	262	277, 285	+	B					-	+	+	+	+	+	+	+	-	5B, 5D
cfe240	TCC	5	213	209-227	+	B	+				+	+	+	+	+	+	+	+	+	3A, 5A, 5B, 7D
cfe241	TG	8	168	186	+	B	+		+		+	+	-	-	-	-	-	-	-	5B
cfe242	GGC	5	218	293-360	+	B	+			+	+	+	+	-	-	-	-	+	-	1A, 1B
cfe243	(CCA)CC(CCA)	4+4	303	339-289	+	D					-	+	-	-	-	-	-	+	-	-
cfe244	CAC	4	170	184	+	B		+			+	-	-	-	+	-	-	-	-	4D
cfe245*	AC	12	239	464	+	A					-	+	-	+	-	-	-	-	-	6B
cfe246	GAA	5	300		-															

cfe247	GCG	6	126	132, 136	+	B	+				+	+	+	+	+	+	+	+	4D, 5A, 5D
cfe248	CCA	4	202	184, 215	+	B					-	+	+	+	+	+	+	-	7A
cfe249	GCT	7	271	490	+	C					-	-	-	-	-	-	-	-	-
cfe250*	CCG	5	127	141	+	A					-	+	+	-	-	-	-	-	4A
cfe251	GCC	6	201		-														
cfe252	GGA	5	131	144, 147	+	B	+				+	+	+	+	+	-	-	-	7B, 7D
cfe253	ACG	6	147	278	+	C	+		+	+	+	+	+	+	+	+	-	-	7A, 7B, 7D
cfe254	GTA	7	259	279-292	+	C	+		+	+	+	+	+	+	-	-	-	-	1B, 4A
cfe255*	CCA	4	146	157, 159	+	A					-	+	+	+	+	+	+	-	1B, 1D
cfe256	CA	12	285		-														
cfe257*	AGC	7	265	272, 274, 280	+	A		+	+	+	+	+	+	+	+	+	+	-	1A, 1B, 1D
cfe258*	AAG	4	144	161	+	A		+	+		+	+	+	+	-	+	-	-	3A, 3B
cfe259	TC	25	142		-														
cfe260*	CCT	7	149	252-258	+	A		+	+	+	+	+	+	+	+	+	+	-	7A, 7B, 7D
cfe261	TC	11	248	252-259	+	A		+	+	+	+	+	+	+	+	+	+	+	7A, 7B, 7D
cfe262	CGG	5+6+4	367		-														
cfe263*	AGC	5	174	186	+	A				+	+	+	+	+	+	-	-	-	1A
cfe264*	CCT	4	254	254-259	+	A	+	+		+	+	+	+	+	-	+	-	-	-
cfe265	AAGGG	4	304	339	+	B		+			+	+	+	-	+	+	-	-	-
cfe266*	ACC	4	251	266	+	A					-	+	+	+	+	+	+	+	1B
cfe267*	GGA	6	172	128, 133	+	A				+	+	+	+	+	+	+	+	+	1A
cfe268	CCT	5	207		-														
cfe269	GCT	4	229		-														
cfe270*	GTG	7	126	141-145	+	A					-	+	+	+	+	+	+	-	4D, 5A, 5B
cfe271*	CGG	5	139	154	+	A					-	+	+	-	-	+	-	-	1B
cfe272*	GCA	4	132	147	+	A					-	+	+	+	+	+	+	+	-
cfe273*	GGC	6	111	**334**	+	A	+	+	+		+	+	+	+	-	+	-	-	6A
cfe274*	CCA	5	180	195-197	+	A				+	+	+	+	+	+	+	-	-	4BL
cfe275	GGA	4	180		-														
cfe276*	AGG	6	125	137, 143	+	A					-	+	+	+	-	+	+	-	1A, 1B
cfe277*	ACA	4	202	208-216	+	A	+		+	+	+	+	+	+	+	+	+	-	6D
cfe278*	CA	12	178	195-212	+	A		+		+	+	+	+	+	+	+	+	-	3B, 7D
cfe279*	CTT	4	246	264	+	A					-	+	+	+	-	+	+	-	6B
cfe280	CGC	4	167		-														
cfe281*	ACG	5	215	228	+	A					-	+	-	+	+	-	-	-	2D, 5D
cfe282*	GAC	7	149	156, 163, 172	+	A	+	+		+	+	+	+	+	+	+	+	+	3A, 3B, 3D
cfe283	CCCG	4	247	186	+	C			+		+	+	+	+	+	+	+	+	2AL, 2B
cfe284*	AATC	4	240	114, 258	+	A					-	+	+	+	+	-	-	+	7A
cfe285*	TGC	7	114	130	+	A					-	+	+	+	+	-	+	+	-
cfe286	AGG	15	187	201	+	B	+	+	+		+	+	+	+	+	+	+	+	2B, 3A, 3D
cfe287	GGC	7	234	224	+	C					-	-	+	-	-	-	-	-	3A
cfe288*	CTC	5	210	320-327	+	A			+	+	+	+	+	+	+	+	+	+	6B
cfe289	CAG	12+4	191		-														
cfe290	CGC	4	132		-														
cfe291	AATC	6	118	134	+	C		+	+		+	+	+	+	+	-	-	-	4D
cfe292	AAC	4	316		-														
cfe293	CCT	4	227	224	+	B	+				+	-	-	+	-	-	-	-	-
cfe294*	AGC	4	250	260	+	A	+				+	+	+	+	+	+	+	-	3D
cfe295	CAG	7	179	174, 184, 194,	+	B	+				+	+	+	+	+	-	-	+	-	3A, 3D, 5D
cfe296	CGC	4	244		-														
cfe297*	CGG	4	150	286, 291	+	A					-	+	+	+	+	+	+	+	+	6A, 6B
cfe298	GAC	7	200																
cfe299	GGA	4	113	249-253	+	B		+			+	+	+	+	+	+	+	+	+	4A, 5A, 5D
cfe300*	AC	13	249	265	+	A	+			+	+	+	+	+	+	+	+	+	+	4A
cfe301	AC	15	177	188-194	+	B	+	+	+	+	+	+	+	+	+	+	+	+	+	5D
cfe302*	GCA	6	283	295-303	+	A	+			+	+	+	+	-	+	-	-	-	-	3B

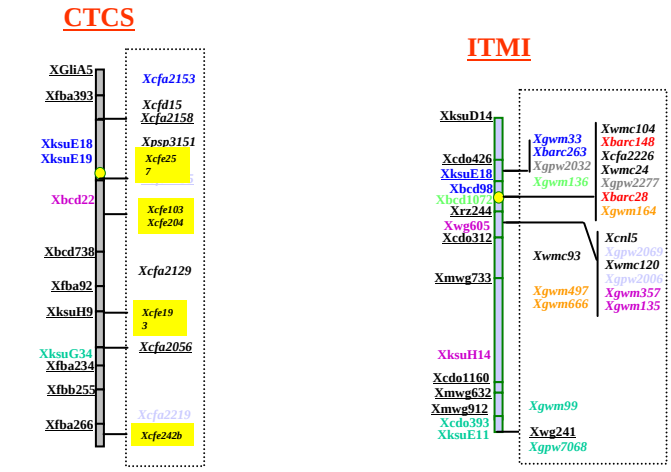
"+" means either polymorphism or amplification. "–" means absence of either polymorphism or amplification.

The PCR product qualities were represented by letters A, B, C and D. A means that the amplification product is strong and clear; B means strong but no clear band; C means weak, but able to be scored and D means too weak to be scored.

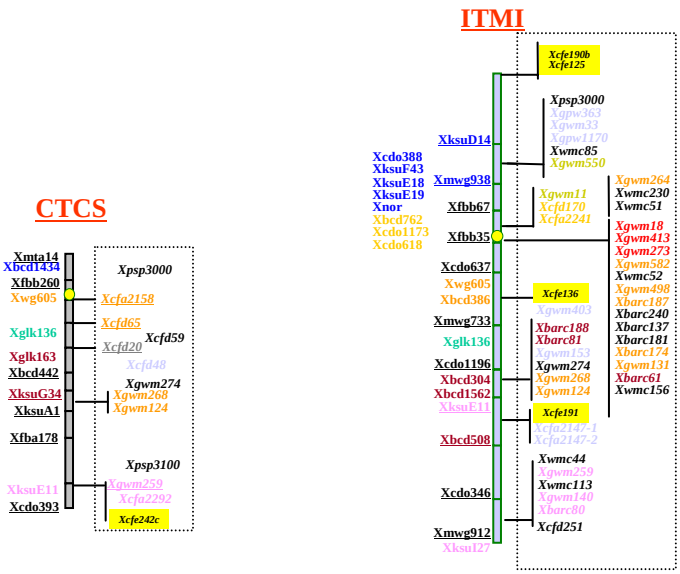
Td: *T. durum*; Tm: *T. monococcum*; As: *Ae. speltoides*; At: *Ae. tauschii*; Sc: *Secale cereale* (rye); Hv: *Hordeum vulgare* (barley); B: *Brachypodium* ; Os: *Oryza sativa* (rice).

Annex 5

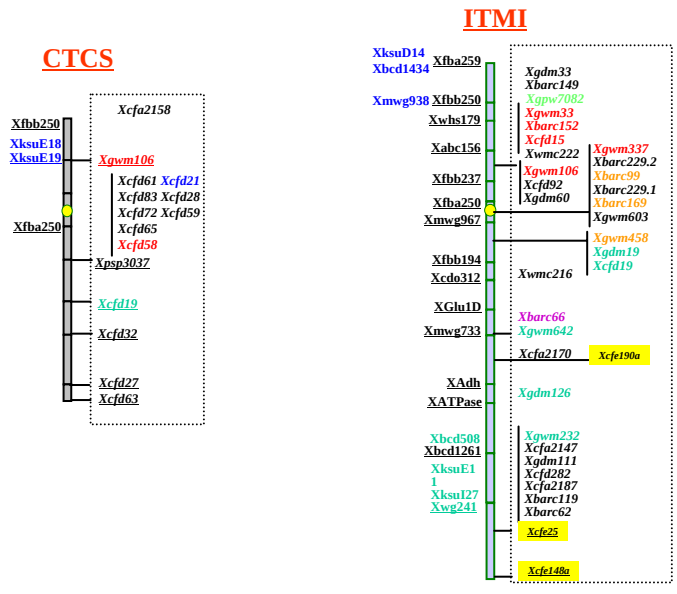
Genetic map in ITMI and CTCS



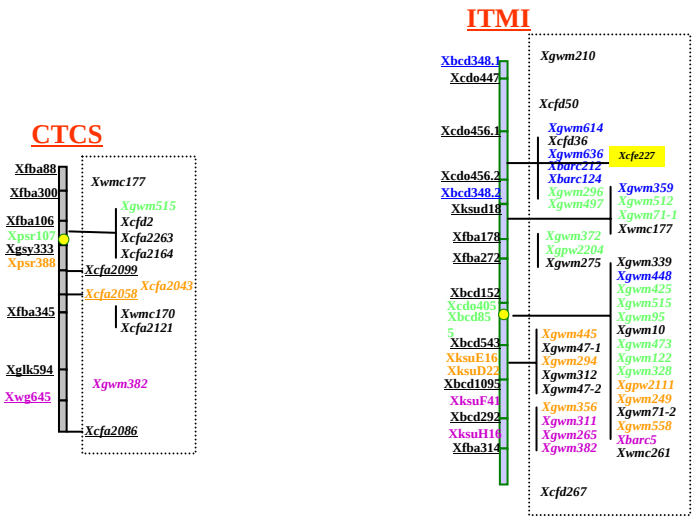
CHROMOSOME 1A



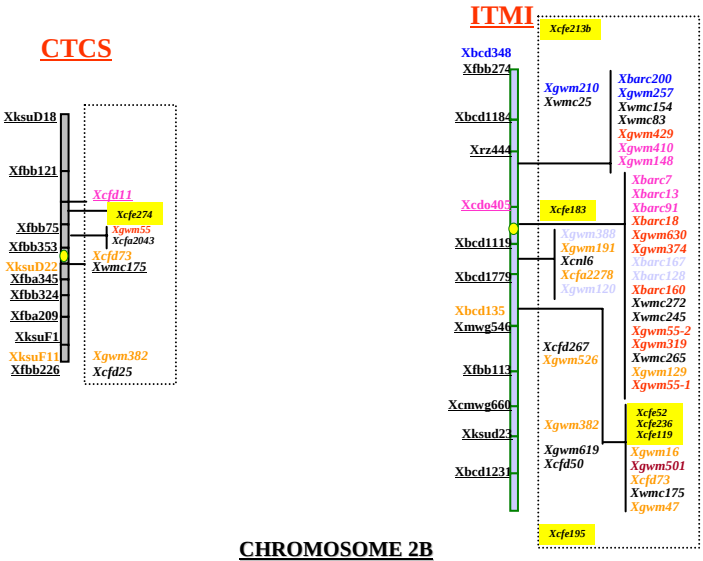
CHROMOSOME 1B



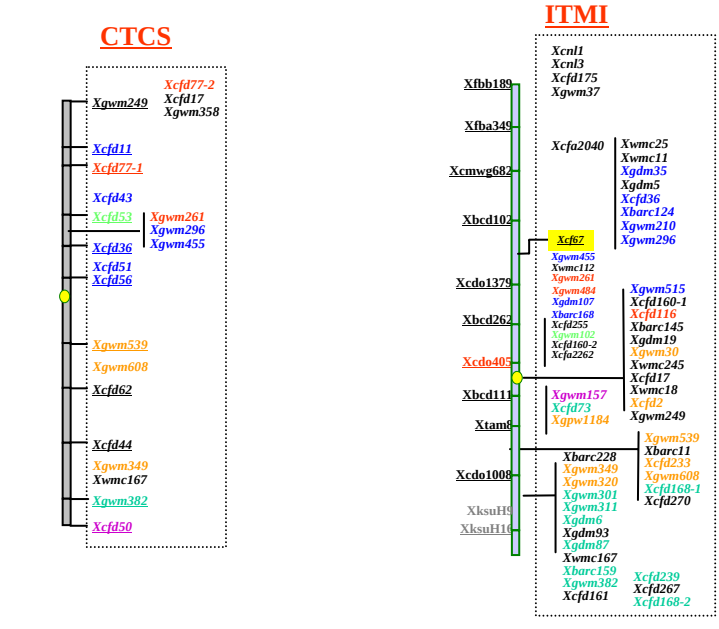
CHROMOSOME 1D



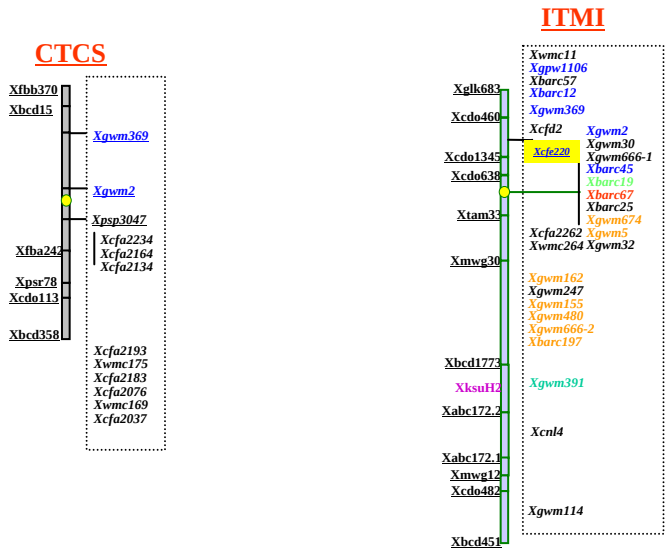
CHROMOSOME 2A



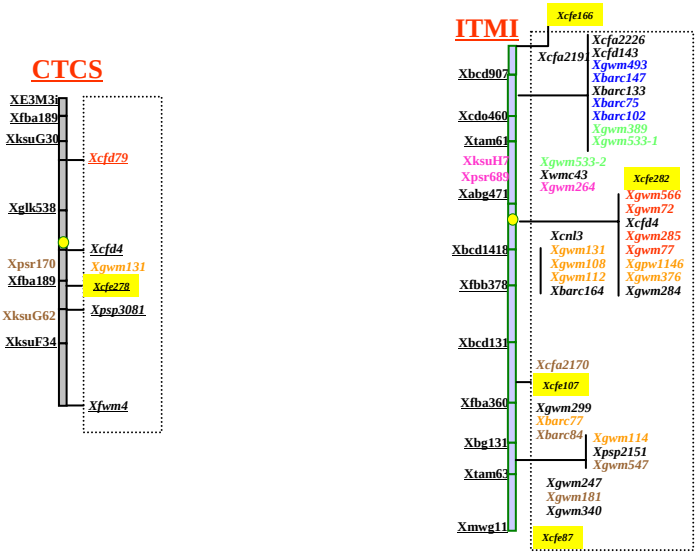
CHROMOSOME 2B



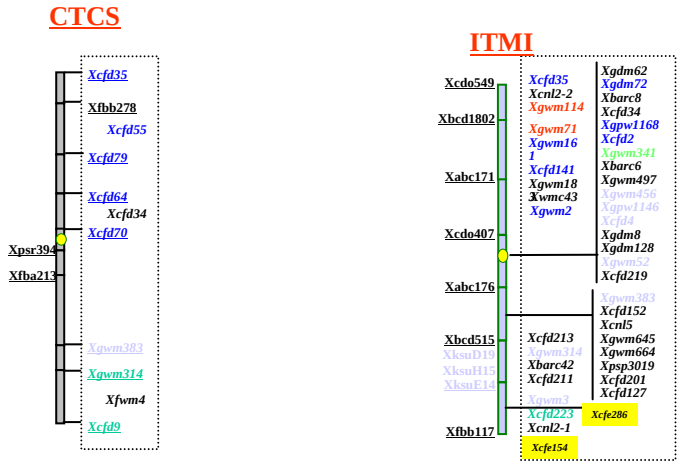
CHROMOSOME 2D



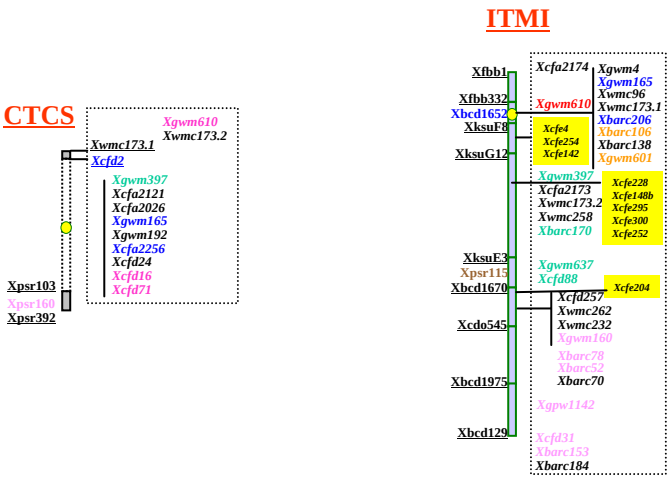
CHROMOSOME 3A



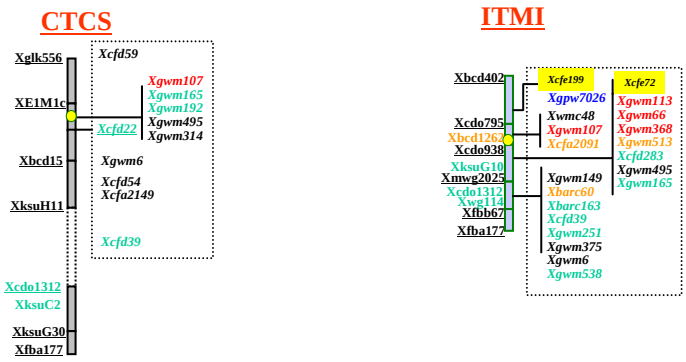
CHROMOSOME 3B



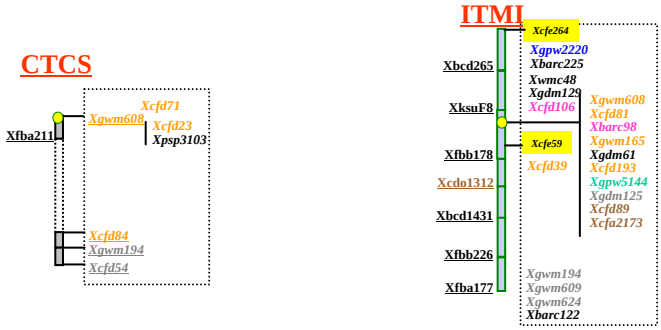
CHROMOSOME 3D



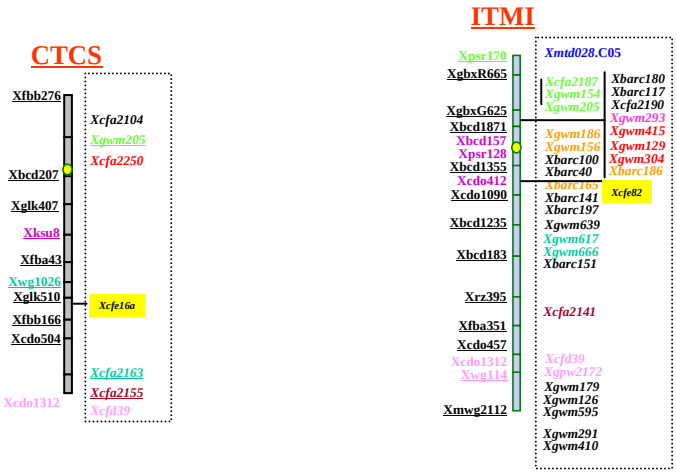
CHROMOSOME 4A



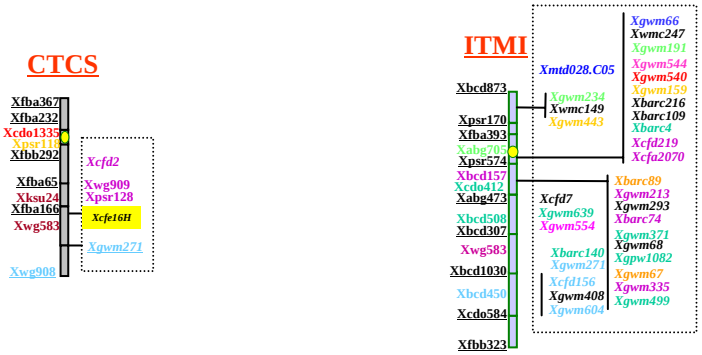
CHROMOSOME 4B



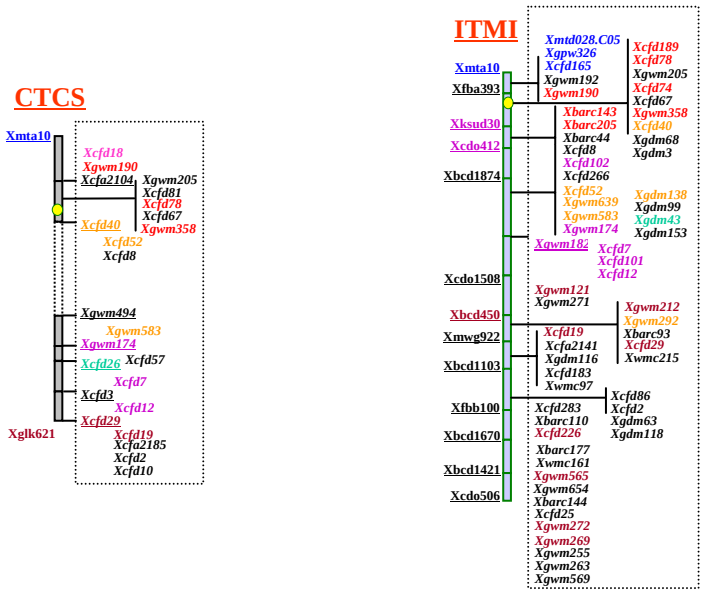
CHROMOSOME 4D



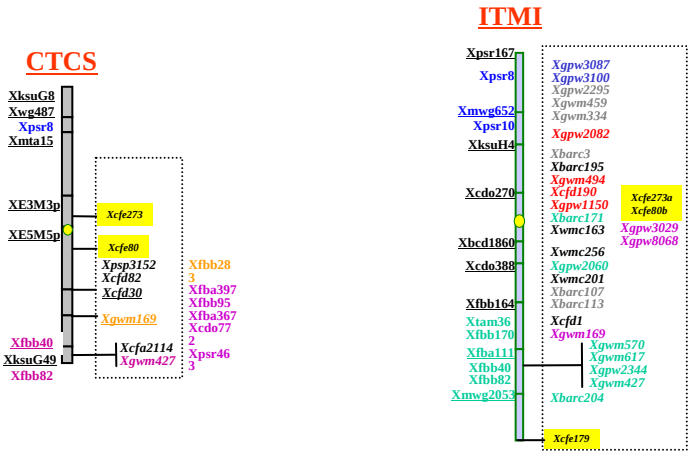
CHROMOSOME 5A



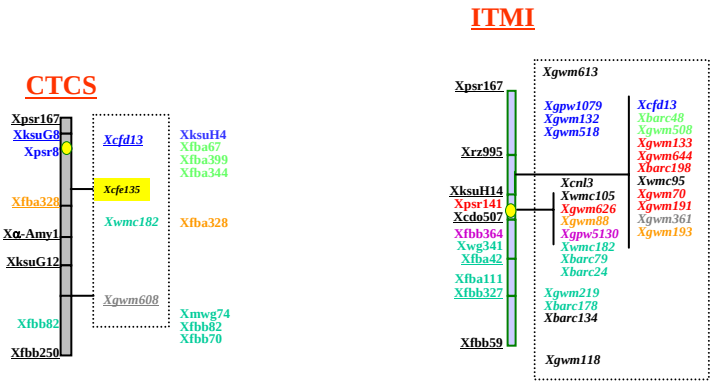
CHROMOSOME 5B



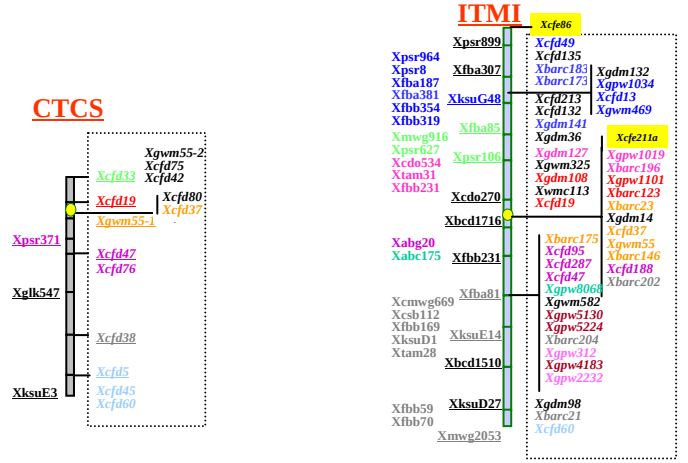
CHROMOSOME 5D



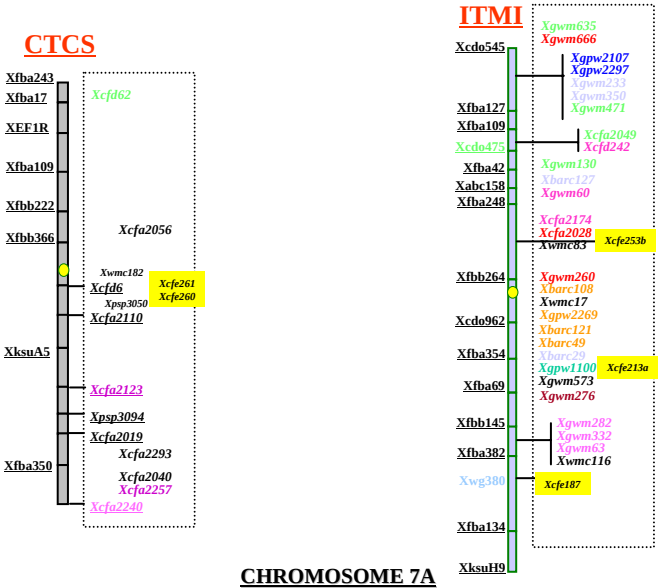
CHROMOSOME 6A



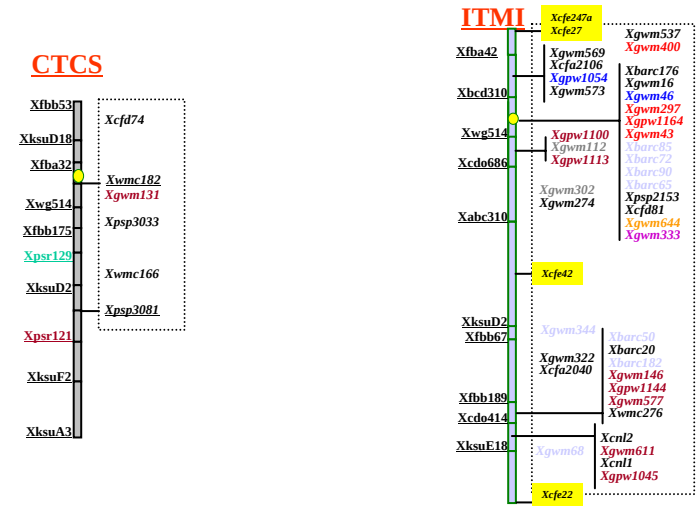
CHROMOSOME 6B



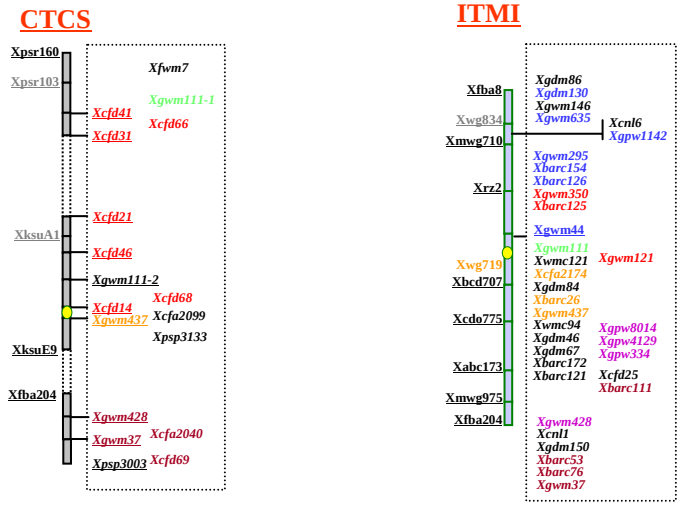
CHROMOSOME 6D



CHROMOSOME 7A



CHROMOSOME 7B



CHROMOSOME 7D

Annex 6

Protocol & Products

Plant DNA extraction using CTAB protocol

This protocol can be applied to triticineae species as well as other species such as maize, *Arabidopsis*, sunflower, barley, rapeseed, tomato, etc. The DNA yield for the triticineae is about 150-200 µg of DNA by gram of fresh material. DNA is of good quality and can be easily measurable through spectro-photometry and digestible by restriction enzymes. Composition of the buffers and solutions are given at the end of the protocol.

Between 0.5 and 3g of young fresh leaves or etiolated coleoptiles are harvested and freezed in liquid nitrogen. At this step, samples can be either stored in deep freezers (-80°C) or directly ground in liquid nitrogen using a mortar and a pestle. The powdered samples are then transferred into individual tubes where 7.5 mL of preheated (65°C) extraction buffer (CTAB 2X) are added. After homogenization, the samples are incubated at 65°C for 30 min with two homogenizations during incubation. Seven mL of either chloroform or dichloromethane supplemented with isoamyl alcohol (24/1) are added and gently mixed for few minutes. The tubes are then centrifuged 15 min (7,500 rpm at 4°C) and the supernatant recovered in a new tube. About 0.1 volume of preheated (65°C) CTAB 10X buffer are then added and mixed before addition of 7 mL of either chloroform or dichloromethane supplemented with isoamyl alcohol (24/1). After homogenization, the tubes are centrifuged 10 min (7,500 rpm at 4°C) and the supernatant recovered in a new tube. Two volumes of precipitation buffer (CTAB P) are then added and mixed gently until a DNA pellet appears. The tubes are then centrifuged 20 min (9,000 rpm, 4°C) and the supernatant discarded. The pellet is dissolved in 3 mL of preheated buffer (TE high). DNA is then precipitated by adding two volumes of cold (-20°C) ethanol, recovered using a glass hook and after a quick rinse in 70% cold ethanol, let dissolve one night at 4°C in 500 µL of 0.1X TE.

After complete re-suspension, DNA is purified by adding 100 µg of RNAse A and incubating 30 min at 37°C. One volume of a solution of phenol/chloroform/isoamyl alcohol (25/24/1) is then added and mixed gently for 2 min. After centrifugation (10 min, 12,000 rpm, room temperature) the supernatant is recovered in a new tube containing one volume of chloroform/isoamyl alcohol (24/1) and gently mixed for 2 min. The tubes are then centrifuged (10 min, 12,000 rpm, room temperature) and the supernatant is recovered in a new tube containing 50 µmoles of sodium chloride. DNA is then precipitated by adding two volumes of cold (-20°C) ethanol, recovered using a glass hook and after a quick rinse in 70% cold ethanol, let dissolve one night at 4°C in 250 µL of 0.1X TE.

DNA concentration can then be measured using a spectro-photometer and adjusted at the desired concentration. Quality of the DNA is checked on a 0.8% agarose gel in TAE 1X buffer.

Buffers and solutions used in the CTAB protocol

- **Chloroform/Isoamyl alcohol (24:1)**

  - chloroform 96%
  - isoamyl alcohol (also methyl-3, butanol-1) 4%

- **CTAB 10%:**

  - CTAB 10 %
  - NaCl 0.7 M

- **Extraction buffer (CTAB 2X):**

  - CTAB 2 %
  - Tris 100 mM
  - EDTA 20 mM
  - NaCl 1.4 M
  - PVP 40 1 %

- **Precipitation buffer (CTAB-P):**

  - CTAB 1 %
  - Tris 0.7 M
  - EDTA 10mM

- **TE 1X (pH 8.0):**

  - Tris 10 mM
  - EDTA 1 mM

For TE 0.1X, dilute ten times.

- **TE High (pH 8.0)**

  - Tris 10mM

- EDTA 50mM
- NaCl 500mM

- **Phenol/chloroforme/isoamyl alcohol (25/24/1)**
  - Phenol 50%
  - Chloroform 48%
  - Isoamyl alcohol 2%
- **RNAse A:**
  - Dissolution buffer (pH 7.5):
  - Tris 10 mM
  - NaCl 15 mM

Add RNAse A at the appropriate concentration and boil 15 min at 100 °C. Store at -20°C

- **TAE 50X (pH 8.0):**
  - Tris 2 M
  - EDTA 50 mM
  - Acetic acid 1 M

Résumé

Bien que la génomique du blé tendre (*T. aestivum* L.) a connu un essor important ces cinq dernières années, peu d'efforts ont été faits dans le domaine de la génomique des espèces sauvages apparentées au blé.

Le premier objectif de cette thèse a été de développer de nouveaux marqueurs moléculaires utilisables sur le blé et transférables vers un nombre important d'espèces cultivées ou sauvages de graminées. Pour cela, nous nous sommes intéressés aux microsatellites localisés dans des séquences de gènes exprimés (EST) afin d'exploiter le nombre important d'EST de blé présentes dans les bases de données et parce que les gènes sont les régions du génome les mieux conservées entre les différentes espèces. A partir d'environ 1 000 contigs d'EST portant un microsatellite nous avons constaté que les motifs tri nucléotidiques étaient les plus fréquents (~80%). Nous avons développé 301 couples d'amorces (EST-SSR) que nous avons testés sur huit variétés de blé tendre et huit espèces apparentées. Environ 80% d'entre eux ont donné un produit d'amplification chez le blé, celui-ci étant en général de grande qualité et polymorphe dans 25,4% des cas (nombre moyen d'allèles = 3,1/locus, valeur PIC moyenne = 0,40). Sur les 240 EST-SSR amplifiables, 177 ont été assignés aux chromosomes de blé grâce aux lignées aneuploïdes et 81 nouveaux locus ont été intégrés aux cartes génétiques de référence. La portabilité des EST-SSR vers les autres espèces apparentées est excellente pour les sous espèces de blé (100% pour *T. aestivum ssp compactum*) et diminue avec l'éloignement phylogénétique pour atteindre encore 28% avec le riz. D'un point de vue évolutif, la projection des séquences d'EST sélectionnées sur la séquence du génome de riz montre une localisation préférentielle près des télomères et une meilleure similarité entre ces deux espèces au niveau des chromosomes 2, 3 et 5 de riz. De même, lorsque l'on s'intéresse aux microsatellites donnant un produit d'amplification à la fois chez le blé, l'orge et le riz, nous remarquons que le motif existe et est fréquemment similaire chez les trois espèces, ce qui suggère une origine commune. D'un point de vue de la diversité allélique des EST-SSR chez les espèces apparentées, nous montrons qu'elle est plus importante chez les espèces allogames telles que *Ae. speltoides*, le ray-grass ou le maïs (respectivement 0,423, 0,388 et 0,352) alors que les espèces autogames comme le blé tendre ou dur montrent des valeurs plus faibles (respectivement 0,108 et 0,093). Le développement réciproque d'une centaine (106) d'EST-SSR à partir de gènes du chromosome 1 de riz donne des résultats de portabilité vers le blé tendre similaires aux précédents (28,5%) et confirme les relations de colinéarité connues entre le chromosome 1 de riz et les chromosomes du groupe d'homéologie 3 du blé.

Le deuxième objectif de cette thèse était d'exploiter les EST-SSR montrant une bonne portabilité pour valider leur capacité dans le cadre d'analyses phylogénétiques chez les Triticées et les graminées. Les résultats indiquent que ces marqueurs permettent de classer les lignées et les espèces conformément aux connaissances concernant la phylogénie et les pedigrees. Ils confirment également que les espèces *T. monococcum ssp urartu*, *Ae. speltoides* et *Ae. tauschii* sont respectivement apparentées aux donneurs des génomes A, B et D du blé tendre.

Nous pouvons donc conclure que les EST-SSR sont des marqueurs intéressants et puissants pour étudier les espèces sauvages orphelines et pour faire des analyses de génomique comparative au sein de la tribu des Triticées et plus généralement de la famille des graminées.

Abstract

Despite recent progress in wheat (*T. aestivum* L.) genomics, only few efforts have been made in the genomics of wild and closely related wheat species.

The first aim of this PhD was to develop new molecular markers useful on wheat and transferable to other cultivated as well as wild grass species. We focused our work on microsatellites (SSRs) located in expressed gene sequences (ESTs) to exploit the large number of wheat ESTs present in the databases and also because the genes are the best conserved regions of the genome between the different species. From about 1,000 EST contigs bearing a microsatellite, we observed that the trinucleotide motifs were the most frequent (~80%). We developed 301 primer pairs (EST-SSRs) that were evaluated on a set of eight wheat cultivars and eight related species. About 80% of the primer pairs gave an amplification product on wheat. Most of the time, we had high quality patterns. The level of polymorphism was estimated to be 25.4% on wheat (mean of 3.1 allele/locus and PIC value of 0.40). Among the 240 EST-SSRs that gave an amplification product on wheat, 177 were assigned to wheat chromosomes using aneuploid lines and 81 new loci were added and integrated to the reference wheat genetic maps. Transferability of wheat EST-SSRs to related species was very good for wheat subspecies (100% for *T. aestivum ssp compactum*) and decreased while the phylogenetic distance increased. However, transferability reached 28% with rice. The alignment of the selected wheat EST-SSRs on the rice genome sequence showed a preferential localisation in the telomeric regions and a better similarity of wheat ESTs on rice chromosomes 2, 3 and 5. Moreover, a thorough study of the EST-SSRs giving an amplification product in wheat, rice and barley indicates that the motif is present and is frequently similar in the three species suggesting a common ancestral origin. The allelic diversity of the EST-SSRs among the wheat related species was higher in allogamous species such as *Ae. speltoides*, *Lolium perenne* and maize (respectively 0.423, 0.388 and 0.352) while autogamous species such as bread and durum wheats showed lower values (respectively 0.108 et 0.093). Reciprocal development of 106 rice EST-SSRs originating from genes of the rice chromosome 1 gave similar results concerning the transferability to wheat (28.5%) as those observed for wheat EST-SSRs and confirmed the known syntenic relationships between rice chromosome 1 and wheat homoeologous group 3.

The second aim of the PhD was the exploitation of the EST-SSRs showing a good transferability to validate their capacities for phylogenetic analyses among the Triticeae and the grasses. The results indicate that these markers are able to properly classify the lines and the species according to the known data about their phylogeny and their pedigrees. The results also confirmed that *T. monococcum ssp urartu*, *Ae. speltoides* and *Ae. tauschii* species are closely related to the ancestral donors of the A, B and D genomes of bread wheat respectively.

Thus, we can conclude that wheat EST-SSRs are interesting and powerful markers to study wheat related orphan species and to make phylogenetic and comparative genomics analyses among the Triticeae tribe and more generally among the grass family.